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The Nucleus-Localized Epidermal Growth Factor Receptor Is SUMOylated

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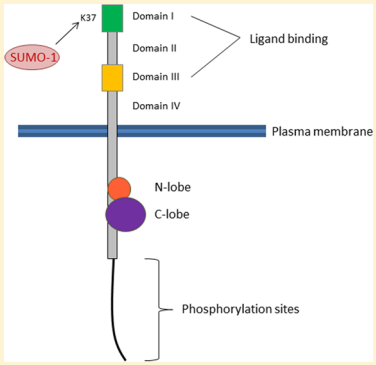
Supporting Information

ABSTRACT: The epidermal growth factor receptor (EGFR) plays important roles in normal and cancer cell growth. The EGFR has principally two different signaling pathways: the canonical kinase route induced at the plasma membrane resulting in an intracellular phosphorylation cascade via MAPKs and PI3K and the more recently discovered pathway by which the receptor functions as a transcriptional co-activator inside the cell nucleus. Full length EGFR translocates to the inner nuclear membrane, via the endoplasmic reticulum, through association with the sec61β translocon. The c-myc (*MYC*) and cyclin D1 (*CNND1*) genes represent two target genes for nuclear EGFR (nEGFR). Here we show that EGFR is SUMOylated and that the SUMO-1-modified receptors are almost unexceptionally nuclear. Co-immunoprecipitation experiments suggest that EGFR is multi-SUMOylated. Using two mass spectrometry-based strategies (matrix-assisted laser desorption ionization time of flight and electrospray ionization liquid chromatography with tandem mass spectrometry), lysine 37 was identified as a SUMO-1-modified residue by both methods. A lysine 37 site mutant (K37R) was transfected into EGFR deficient cells. Total SUMOylation of EGFR was not altered in the K37R-transfected cells, confirming the presence of other SUMOylation sites. To gain preliminary insight into the possible functional role of EGFR SUMOylation, we compared the effect of expression of the wild-type EGFR with the K37R mutant on promoter activity and expression of *CMYC* and *CNND1*. Our results indicate that SUMO-1 modification may affect the transcriptional activity of EGFR, which might have additional impact on, e.g., cancer progression.



The epidermal growth factor receptor (EGFR) is one of four members of the ErbB/HER family of transmembrane receptor tyrosine kinases (RTKs).[1] EGFR activates downstream signaling through pathways such as MAPK/Ras, PI(3)K/Akt, and PLCγ/PKC, pathways that play a central role in cell survival and progression of cancer cells.[2,3] The EGFR was one of the first RTKs observed in the cell nucleus.[4,5] Today, it is known that several other RTKs can also be translocated to the cell nucleus.[6−12] Nuclear EGFR functions as a transcriptional co-activator for several proteins, including cyclin D1,[13] c-myc,[14] and iNos.[15] Furthermore, it has been suggested to be involved in DNA repair through stabilization of PCNA[16] and DNA-PK.[17]

The small ubiquitin-like modifier (SUMO) is a post-translational modification that can regulate subcellular localization, DNA repair, protein−protein interactions, stability, and gene transcription.[18−22] Recently, the insulin-like growth factor 1 receptor (IGF-1R) and ErbB-4 were shown to be modified by SUMO-1, and upon SUMOylation, the receptors become stabilized and translocated into the cell nucleus.[8,23,24]

In this study, we first demonstrated that the EGFR is SUMOylated by SUMO-1 as well as SUMO-2/3 and that the SUMO-1-modified receptor is localized mainly in the cell nucleus. We identified lysine 37 of the receptor as one of the targets for SUMO-1 modification. SUMOylation of nEGFR may have a functional role in transcriptional transactivation.

MATERIALS AND METHODS

Reagents. Antibodies against EGFR (catalog no. 377229), GAPDH (catalog no. 25778), and IgG (catalog no. 2025) were obtained from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Mouse anti-EGFR (catalog no. 610016) was

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purchased from BD Biosciences (Stockholm, Sweden). EGFR (catalog no. 2232), pEGFR (catalog no. 3777), c-myc (catalog no. 5605), Myc-tag (catalog no. 2368), histone H3 (catalog no. 9715), Na,K-ATPase (catalog no. 3010), cyclin D1 (catalog no. 2922), pAkt (catalog no. 4060), pErk (catalog no. 4370), SUMO-1 polyclonal (catalog no. 4930), and SUMO-1 monoclonal (catalog no. 4940) antibodies were purchased from Cell Signaling Technology, BioNordika (Stockholm, Sweden). The Alexa Fluor 488-conjugated goat anti-mouse antibody was purchased from Molecular Probes, Life Technologies (Stockholm, Sweden). All other reagents were from Sigma-Aldrich (Stockholm, Sweden), unless otherwise stated.

Cell Culture and Transient Transfections. H1299, HCT116, and HeLa cells were purchased from LGC Standards (Borås, Sweden) and maintained in RPMI-1640, McCoy's 5a Medium Modified, and Eagle's Minimum Essential Medium, respectively (Life Technologies). PAE cells were a kind gift from A. Östman (Karolinska Institute) and cultured in Dulbecco's modified Eagle's medium/F12 (Life Technologies). All media were supplemented with 10% FBS. PAE cells were transiently transfected with Fugene HD from Promega Biotech AB (Stockholm, Sweden). His-SUMO-1 (catalog no. 17271), GFP-SENP2 (catalog no. 13382), and Ubc9 (catalog no. 14438) expression vectors were purchased from Addgene (Cambridge, MA). EGFR-K37R and Myc-T95R-SUMO1 were generated by using the Quick-Change site-directed mutagenesis kit (Agilent Technologies, Kista, Sweden). EGF was obtained from BD Biosciences, and the stimulation experiment was conducted using 100 ng/mL EGF for 5 min, unless otherwise stated.

Immunoblotting and Immunoprecipitation. For non-denatured IPs, the cells were harvested in modified RIPA lysis buffer [50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholoate, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 20 mM *N*-ethylmaleimide, and 1× protease and phosphatase inhibitors]. For subcellular IPs, fractions were isolated with the Qproteome Cell Compartment Kit (Qiagen, Sollentuna, Sweden) and diluted in a 1:10 ratio with modified RIPA lysis buffer. For denatured IP experiments, 10^7 cells were boiled in 100 μL of TSD buffer (50 mM Tris-HCl, 1% SDS, 5 mM DTT, 20 mM *N*-ethylmaleimide, and protease and phosphatase inhibitor) for 10 min before being sonicated briefly and centrifuged at 16000*g* for 10 min. The supernatants were then diluted with 1.2 mL of TNN buffer (50 mM Tris-HCl, 250 mM NaCl, 5 mM EDTA, 0.5% NP-40, 20 mM *N*-ethylmaleimide, and 1× protease and phosphatase inhibitor). The immunoprecipitations were conducted by incubating the lysates overnight with 5 μL of anti-EGFR (catalog no. 610016) antibody and 10 μL of protein G Dynabeads (Life Technologies) at 4 °C. Precipitated proteins were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (Life technologies), transferred onto a nitrocellulose membrane (GE Healthcare, Uppsala, Sweden), blotted with specific antibodies [EGFR (catalog no. 2232) and SUMO-1 (catalog no. 4940) were used for immunoblotting], and visualized with ECL detection reagents (GE Healthcare).

Dual-Luciferase Promoter Assay. Cyclin D1 and c-myc promoter activities were determined by the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's manual. *Renilla* luciferase vector (pGL4.70[hRluc]) was purchased from Promega. Cyclin D1 luciferase promoter plasmid was a kind gift from D. Grandér (Karolinska Institute), and the c-myc promoter plasmid was purchased from Addgene (catalog no. 14968).[25]

DuoLink *in Situ* Proximity Ligase Assay (PLA). The assay was conducted according to the manufacturer's instructions. Antibodies used were SUMO-1 (catalog no. 4930) and EGFR (catalog no. 610016). Analysis was conducted with a wide-field Zeiss (Oberkochen, Germany) Axioplan2 imaging microscope, at 40× magnification. Images were acquired with an AxioCamHR camera and analyzed in AxioVision 3.1. All images are z-stacked.

Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) Peptide Mass Fingerprinting. wt-EGFR, SUMO-1, and Ubc9 were co-overexpressed in PAE cells that lack endogenous EGFR. EGFR was immunoprecipitated under nondenatured conditions with EGFR antibody (catalog no. 377229) 48 h post-transfection. MALDI-TOF mass spectrometry was performed as described by Zakharchenko.[26] Briefly, after being separated in a NuPAGE 3−8% Tris-acetate gel and stained with the Pierce Coomassie R-250-based Imperial Protein Stain (Thermo Scientific, Rockford, IL), the EGFR bands were excised, destained, and subjected to in-gel digestion with trypsin (Promega, Madison, WI). The concentrated and desalted tryptic peptides were directly eluted with 50% acetonitrile containing α-cyano-4-hydroxycinnamic acid matrix onto the metal target and analyzed by MALDI-TOF MS on a Micromass M@LDI-Reflectron instrument (Waters Corp., Milford, MA). The peptide spectra were internally calibrated using autolytic peptides from trypsin (842.510, 1045.564, and 2211.105 Da), and the +2154.9383 Da mass shift corresponding to the ELGMEEEDVIEVYQEQTGG tryptic peptide from the SUMO-1 modification side chain was used as a signature of SUMOylation. The following parameters were used in the analysis: one miscleavage, maximal mass tolerance of 0.5 Da, and spectral scoring set to $p < 0.05$. The probability of identification (p value) and Z value as additional evaluation of significance were considered, as suggested by the ProFound search engine. PMF searches were performed with the NCBIr database.

Liquid Chromatography with Tandem Mass Spectrometry (LC−MS/MS). PAE cells overexpressing wt-EGFR, T95R-SUMO-1, and Ubc9 were denatured in TSD buffer followed by immunoprecipitation, separation, and staining as described above. Tryptic digestion was performed by a liquid-handling robot.[27] Nano-LC−MS/MS analysis was performed using an Easy-nLC system (Thermo Scientific) directly coupled to a hybrid LTQ Orbitrap Velos ETD mass spectrometer (Thermo Scientific). The MS acquisition method was comprised of one survey full scan at a resolution of R = 60000 (m/z 400), followed by up to 10 data-dependent consecutive electron-transfer dissociation (ETD) and higher-energy collisional dissociation (HCD) MS2 scans or alternatively collision-induced dissociation (CID) scans only. ETD and CID MS2 scans were acquired in profile mode. The data were searched against the human SwissProt protein database (version 2013.04) using the Mascot Deamon 2.3.0 search engine (Matrix Science Ltd., London, U.K.). Post-translational modifications were verified by using Scaffold PTM 1.1.3 (ProteomeSoftware). Identified SUMOylated peptides had an Ascore of 1000.00 and a localization probability of 100%. Parameters were chosen as follows: up to two missed cleavage sites for trypsin, peptide mass tolerance of 10 ppm, and 0.05 Da for the HCD and 0.5 Da for ETD fragment ions. The Mascot confidence interval was set at 29. Carbamidomethylation of

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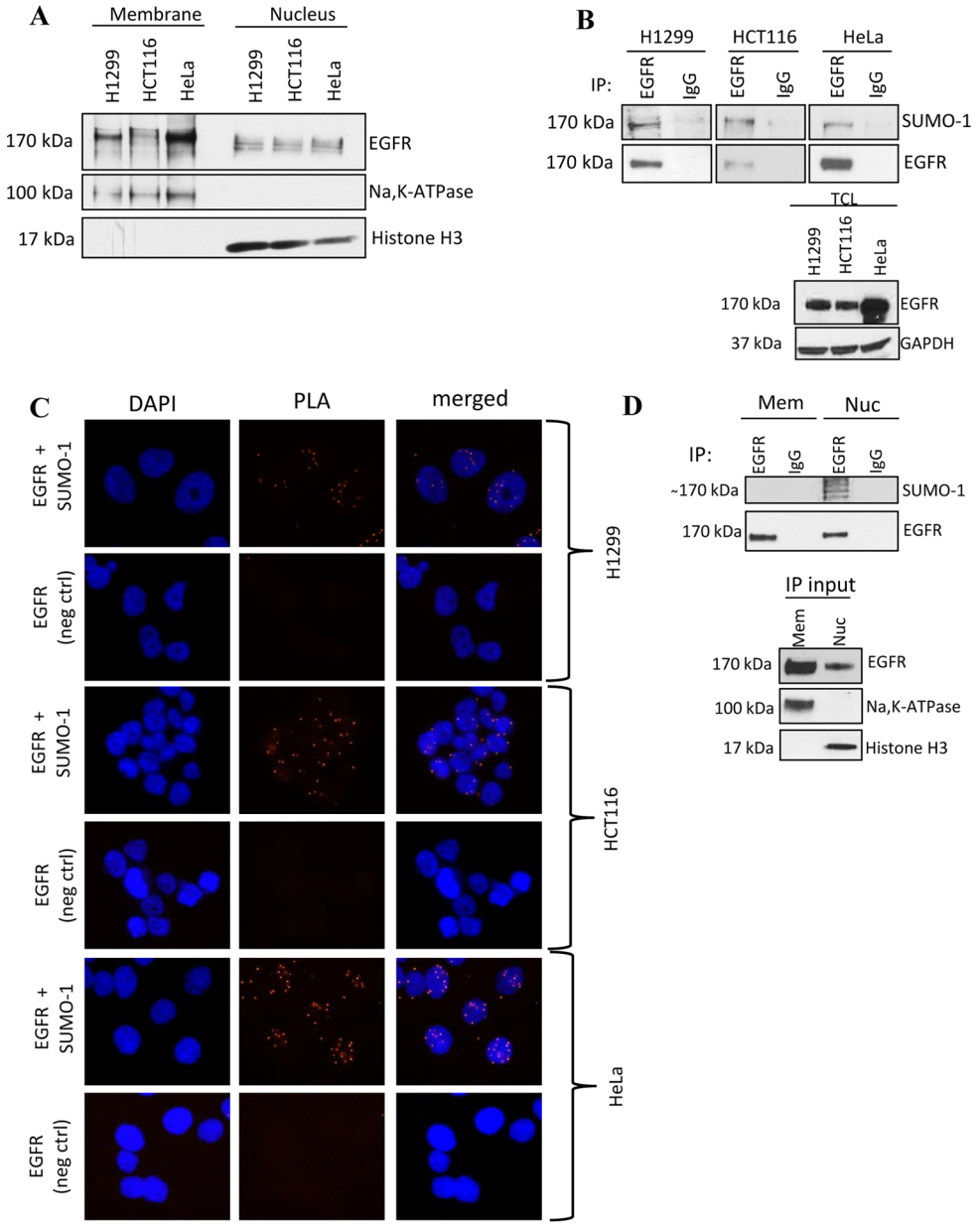

Figure 1. EGFR is SUMOylated in the nucleus. (A) H1299, HeLa, and HCT116 cells were grown under basal conditions followed by subcellular fractionation. Lysates were immunoblotted against EGFR. To verify the purity of the fractions, they were analyzed for the plasma membrane marker Na,K-ATPase and nuclear marker histone H3. (B) Cell lysates from H1299, HeLa, and HCT116 cells were IPed under nondenaturing condition with either EGFR or IgG (negative control) antibody, followed by IB against SUMO-1 and EGFR. Total cell lysates (TCL) showing the total amount of EGFR and GAPDH as a loading control (bottom panels). (C) Colocalization of EGFR and SUMO-1 investigated by a PLA in H1299, HeLa, and HCT116 cells (red dots). Only the EGFR antibody was used as a negative control. (D) H1299 cells were subjected to subcellular fractionation followed by IP with EGFR or IgG antibodies and IB against SUMO-1 and EGFR. The bottom panels show the IP input after fractionation; EGFR in membrane and nuclear fractions and Na,K-ATPase and histone H3 to verify the purity of the fractions.

cysteine was specified as a fixed modification, whereas oxidation of methionine, deamidation of asparagine and glutamine, acetylation of lysine, serine, histidine, tyrosine, and threonine, phosphorylation of serine, threonine, and tyrosine, and SUMOylation/ubiquitylation of lysine were defined as variable modifications.

Statistical Analysis. Protein quantifications of immunoblots were analyzed with ImageJ. Analysis is based on raw data after normalization to controls from three independent experiments. Via a paired two-tailed Student's *t* test, means and the standard deviation (SD) were calculated to verify significance (one asterisk and two asterisks represent $p < 0.05$ and $p < 0.01$, respectively).

RESULTS

EGFR Is SUMOylated. It has previously been shown that IGF-1R and ErbB-4 are SUMOylated by SUMO-1.[8,23] SUMOylated proteins were initially reported to be expressed in the cell nucleus, but later also in other parts of the cell.[18] To investigate whether EGFR is SUMOylated, we used three cancer cell lines: H1299 (non-small cell lung cancer), HCT116 (colon cancer), and HeLa (cervical cancer). In line with previous studies, these cell lines exhibit nEGFR,[28−30] as assayed

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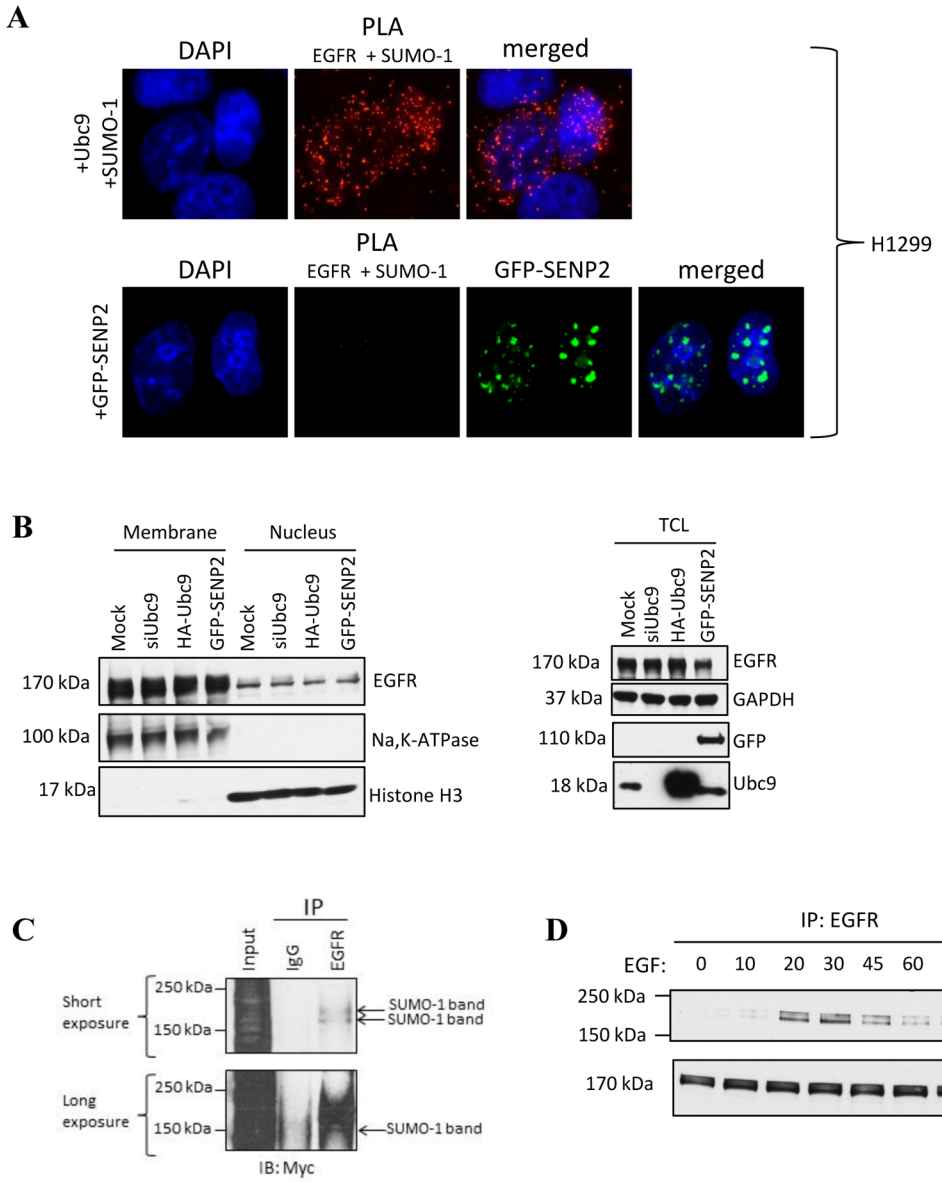

Figure 2. EGFR is SUMOylated at multiple lysine residues. (A) H1299 cells were transfected with either SUMO-1/Ubc9 or GFP-SENP2 followed by PLA between EGFR and SUMO-1 (red dots). (B) Left panels show that cells transfected with mock (empty plasmid), HA-Ubc9, siRNA against Ubc9 and GFP-SENP2 were subjected to subcellular fractionations followed by IB against EGFR, Na,K-ATPase, and histone H3. Right panels show total cell lysates before fractionations to verify transfections. IB against EGFR, GAPDH, GFP, and Ubc9. (C) H1299 cells were transfected with Myc-T95R-SUMO-1/Ubc9 followed by denaturing IP with IgG and EGFR and IB against the Myc tag. (D) H1299 cells serum starved for 24 h post-transfection with Myc-T95R-SUMO-1/Ubc9 for 24 h followed by 100 ng/mL EGF stimulation. Lysates were used for denaturing IP with EGFR and detection of the Myc tag and EGFR.

by subcellular fractionations (Figure 1A). In these experiments, cells were cultured under basal conditions. The purity of the fractionations was confirmed by markers for the plasma membrane (Na,K-ATPase) and the nucleus (histone H3). The EGFR SUMOylation by SUMO-1 was identified by immunoprecipitation (IP) experiments on total cell lysates, with either EGFR or IgG antibody (negative control) followed by detection of SUMO-1 and EGFR (Figure 1B). Colocalization and the subcellular distribution between the receptor and SUMO-1 were assessed by an *in situ* proximity ligase assay (PLA). In brief, cells are grown on immunofluorescent coverslips and incubated with primary antibodies simultaneously, which were raised in different species, e.g., rabbit and mouse. The secondary antibodies are fused with PLA probes, one plus and one minus DNA oligonucleotide strand, which detect anti-rabbit and anti-mouse primary antibodies. If the two proteins of interest are in the proximity of each other, i.e., <40 nm, these probes form a DNA circle that is amplified through a polymerase reaction. As a consequence, each protein interaction results in a bright fluorescent signal. Here, we found that the EGFR and SUMO-1 proteins colocalized inside the nucleus in all three cell lines (Figure 1C). The specificity of the assay was verified by using only the EGFR antibody (negative control). To assess if the EGFR can also be modified by SUMO-2/3, we performed PLA between these proteins, as well, and indeed, the EGFR is also SUMOylated by SUMO-2/3 (Figure S1). Remarkably, this modification is, in contrast to SUMO-1, mainly in the cytoplasm. Because we are interested in

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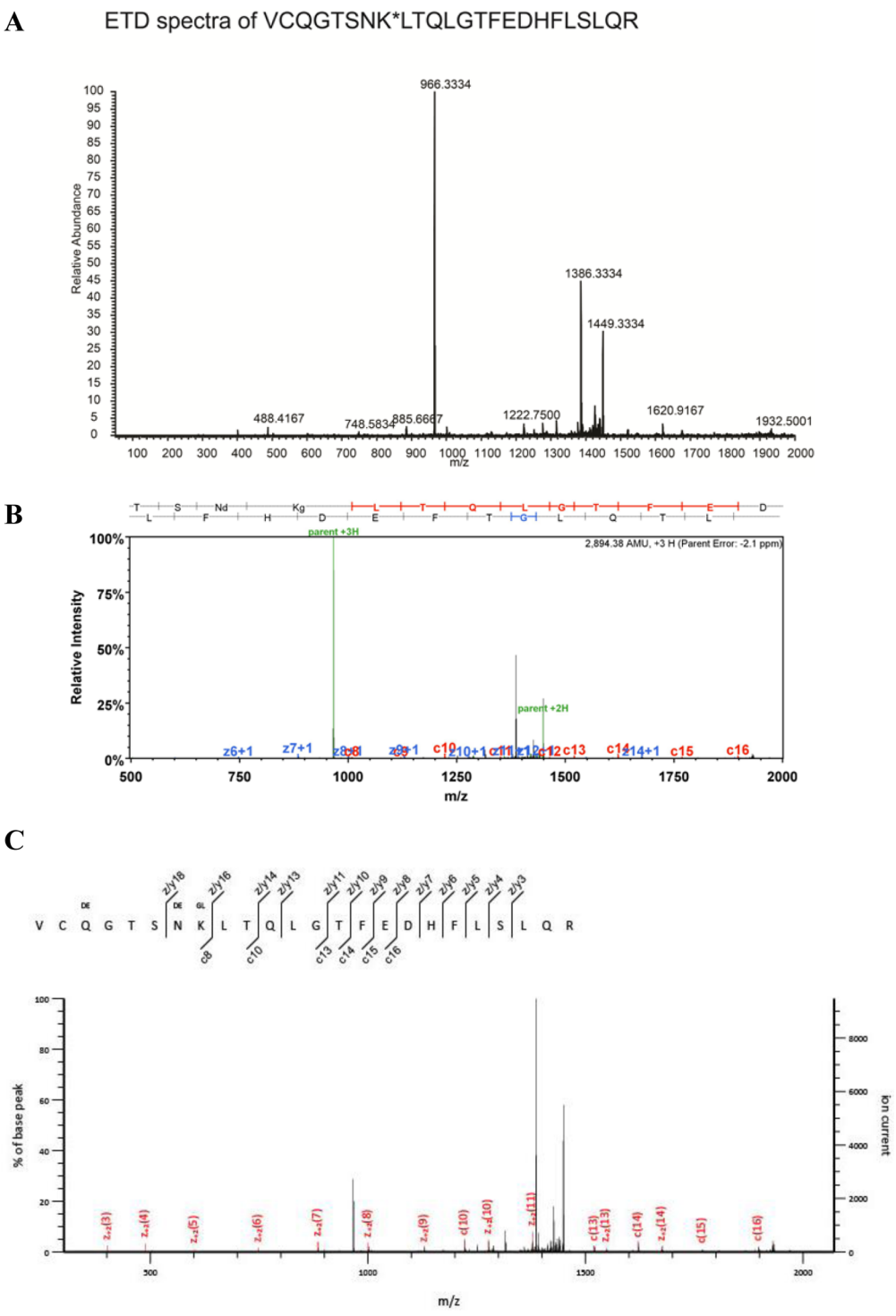

Figure 3. Mass spectrometry (LC–MS/MS) reveals multiple lysine residues as SUMO-1 targets of EGFR. (A and B) Fragmentation spectra of VCQGTSNK*LTQLGTFEDHFLSLQR (K37) in ETD fragmentation mode. (C) Corresponding annotated spectrum exported from Scaffold.

the function of nuclear EGFR in relation to SUMOylation, we decided to focus on only SUMO-1 in this investigation. To further explore the cellular localization of SUMOylated EGFR, H1299 cells were first subjected to subcellular fractionation followed by IP with either EGFR or IgG antibody on membrane and nuclear fractions and detection of SUMO-1 and EGFR. Results confirmed PLA data showing only association between EGFR and SUMO-1 in the nucleus (Figure 1D, top panels). The IP input, membrane and nuclear fractions, was analyzed with antibodies against EGFR, Na,K-ATPase, and histone H3 (Figure 1D, bottom panels).

SUMOylation is homologous to ubiquitylation and requires three enzymatic steps: the ATP-dependent activating enzyme E1, SAE1/SAE2 (also known as Aos1/Uba2), the E2-conjugating enzyme Ubc9, and one of several E3 ligases. The SUMOylation process is reversible, and deSUMOylation of conjugated substrate is mediated by, for example, proteins belonging to the SENP family.[31] To investigate if EGFR SUMOylation is required for its nuclear translocation, we first

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Table 1. Identification of Lysine 37 in EGFR as a Putative SUMO-1 Target via LC−MS/MS

Start	Stop	Peptide Sequence	SUMOylation *	Peptide Score	Mascot:Score	Observed Mass	Charge	Delta PPM	Spectrum Name	Exp
30	53	VCQGTSNKLTQLGTFEDHFLSLQR **	K37	36.7	15.87	965.801	3	-1.97	HCD	1
30	53	VCQGTSNKLTQLGTFEDHFLSLQR **	K37	3.7	35.38	965.801	3	-1.97	ETD	1
38	53	LTQLGTFEDHFLSLQR			95.82	952.9986	2	1.8	HCD	1
38	53	LTQLGTFEDHFLSLQR			69.09	635.6672	3	0.291	HCD	1
38	53	LTQLGTFEDHFLSLQR			53.94	952.9986	2	1.8	ETD	1
38	53	LTQLGTFEDHFLSLQR			43.92	635.6672	3	0.291	ETD	1

overexpressed Ubc9/SUMO-1 or GFP-SENP2 followed by detection of EGFR−SUMO-1 interaction by PLA (Figure 2A). Overexpression of Ubc9/SUMO-1 induced hyper-SUMOylation, and the colocalization was no longer solely nuclear; here EGFR SUMOylation was also found in the cytoplasm. Overexpression of GFP-SENP2 completely abolishes EGFR−SUMO-1 interactions. Next we performed subcellular fractionations after transfections with mock (empty plasmid), knockdown of Ubc9 by siRNA, and overexpression of Ubc9 and SENP2 (Figure 2B, left). Total cell lysates (TCL) were collected prior to fractionations, and transfections were assessed (Figure 2B, right). None of the transfections affected the levels of nuclear EGFR.

All PLAs and IPs from Figure 1 were performed under nondenaturing conditions. To exclude the possibility that the SUMOylation of the EGFR might be due to colocalization of other SUMOylated proteins, we performed denatured IP (by lysing the cells in SDS buffer followed by boiling) with IgG or EGFR antibody in H1299 cells transfected with Ubc9/Myc-SUMO-1 followed by detection of Myc (Figure 2C). The samples were separated on a low-percentage gel, and at least three individual SUMO-1 bands were detected. SUMO-1, in contrast to SUMO-2/3, does not form chains,[32] suggesting that the receptor is SUMOylated at multiple sites. It has previously been shown that nEGFR is ligand-dependent in different cell lines.[13,29,33] Serum-starved H1299 cells have nEGFR, but the level increases after EGF stimulation for 5 min (Figure S3). To determine if EGFR SUMOylation is EGF-dependent, H1299 cells transfected with Ubc9/Myc-SUMO-1 were serum-starved (RPMI medium without serum) 24 h post-transfection for 24 h followed by EGF stimulations for 10−80 min. Denatured IP with EGFR followed by detection of Myc and EGFR shows that EGFR SUMOylation peaks after EGF stimulation for 20−30 min (Figure 2D).

Identification of Lysine 37 as a SUMO-1 Target. Mass spectrometry-based analyses were conducted to identify SUMOylation sites in EGFR (for details, see Materials and Methods). For this purpose, the porcine aortic endothelial (PAE) cell line, lacking endogenous EGFR, was used. wt-SUMO-1 creates a side chain of 19 amino acids on the binding site of the peptides after digestion. Using MALDI-TOF peptide mass fingerprints and ESI LC−MS/MS approaches, we detected potential SUMOylation corresponding to lysine 37 (Figure S2 and Figure 3A−C, respectively). Via LC−MS/MS, we had introduced a T95R point mutation into the C-terminus of SUMO-1 that does not affect the function of SUMO-1 but generates a new cutting site that leaves a Gly-Gly signature on the SUMOylated peptides.[34] After acquisition of the tandem mass spectrometric data, the EGFR peptide VCQGTSNK*LTQLGTFEDHFLSLQR, containing the modified K37, was identified in both ETD and HCD scans (Table 1). Further, LC−MS/MS identification of K37 strengthened MALDI MS data that indicated miscleavage of the K37-SUMOylated peptide [VCQGTSNK(ELGMEEEDVIEVYQEQTGG)]. All these peptide modifications had a localization probability of 100%, which confirmed the accuracy of the site identifications. Both MALDI-TOF and LC−MS/MS pointed to K37 as a SUMOylation site, and K37 was the only lysine residue that passed the preset confidence threshold in our analysis, which justified further study of this site to provide additional proof of SUMOylation.

The EGFR-K37R Mutation Does Not Impair Kinase Activity, Nuclear Translocation, or the Stability of EGFR. Using site-directed mutagenesis with wt-EGFR as a template, a lysine-to-arginine mutant was generated at K37. wt-EGFR, K37R, and mock plasmids were transfected into PAE cells. After 48 h, the cell lysates from the different transfections were subjected to denaturing IP of EGFR followed by detection of SUMO-1 and EGFR. The single mutation of K37 did not block the SUMOylation of EGFR (Figure 4A). This was supported by PLA in PAE cells, showing no difference in EGFR−SUMO-1 interactions between wt-EGFR and K37R. To identify positively transfected cells, the cells were costained against EGFR using Alexa-488 after the PLA protocol was completed (Figure 4B). The PLA interactions were quantified by DuoLink ImageTool, and no significant difference was observed between wt-EGFR and K37R (Figure 4C). This further suggests that EGFR is SUMO-1-modified at several lysine residues.

Lysine 37 is situated in the L1 ligand binding domain of the receptor and therefore we evaluated in terms of whether the K37R mutation affects the downstream activation of EGFR. PAE cells were transiently transfected with the mock, wt-EGFR, or EGFR-K37R plasmid, and after 24 h, the cell medium was replaced with serum free medium and incubated for an additional 24 h. Via stimulation of the cells with EGF, the EGFR-specific phosphorylation of EGFR, Akt, and Erk was assessed by immunoblotting (Figure 4D). Quantification of pEGFR, pErk, and pAkt, after normalization to GAPDH, showed no significant difference in activation between wt-EGFR and K37R (Figure 4E). As expected, no difference was observed in nuclear translocation of wt-EGFR of the K37R mutant (Figure 4F). In other studies, we showed that SUMOylation of IGF-1R affects both the nuclear translocation and the stability of the receptor.[8,24] To assess if the stability of the EGFR could be affected by the point mutation, we transfected cells with the EGFR constructs followed by cycloheximide (CHX) treatment 24 h post-transfection. The cells were treated with CHX for 12 or 24 h. No difference in degradation rate was observed (Figure 4G,H).

An Indication That SUMOylation of EGFR-K37 Affects Transcriptional Activity. SUMO-1 modification is well-documented to affect transcriptional activity. There are several studies describing SUMO-1 as a transcription suppressor, although it has also been reported to function as an activator of gene transcription.[35−40] Because we did not observe any difference in either stability or nuclear translocation, we

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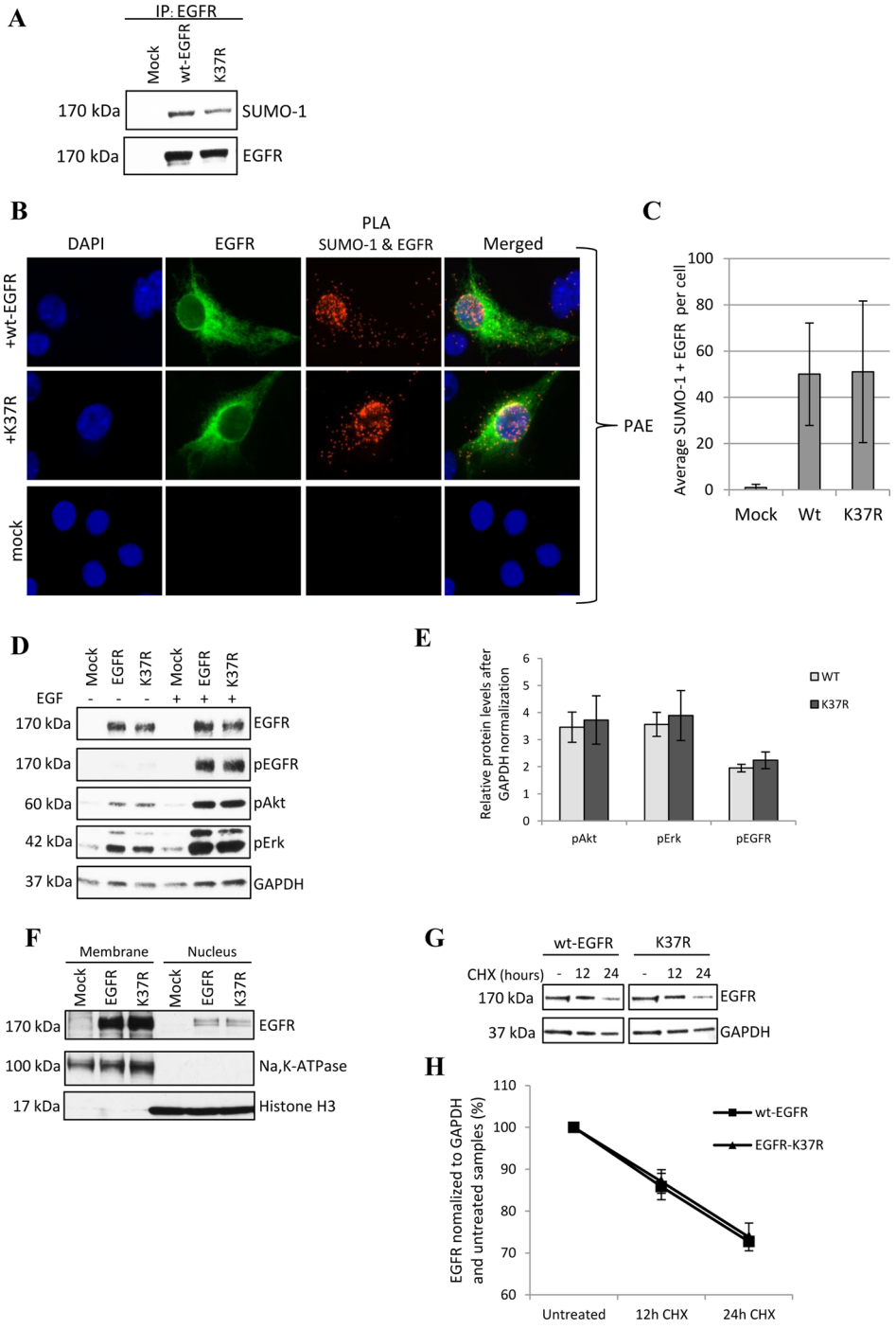

Figure 4. EGFR-K37R mutant does not affect EGFR kinase activity, stability, nuclear translocation, or overall SUMOylation. (A) PAE cells were transfected with the mock, wt-EGFR, or EGFR-K37R plasmid together with Myc-T95R-SUMO-1/UBC9 followed by denaturing IP with anti-EGFR and immunoblotted against the Myc tag, followed by reblotting against EGFR. (B) PLA between EGFR-SUMO-1 (red dots) in PAE cells transfected with with the mock, wt-EGFR, or EGFR-K37R plasmid. The transfected cells were costained against EGFR (green) to identify positively transfected cells. (C) Quantification of PLA interactions in panel B using DuoLink ImageTool. (D) PAE cells were transfected with the mock, wt-EGFR, or EGFR-K37R plasmid. Cells were kept serum-starved for 24 h followed by either 100 ng/mL EGF stimulation for 5 min or mock stimulation. Lysates were analyzed by immunoblotting against EGFR, pEGFR pAkt, pERK, and GAPDH (loading control). (E) Densitometric analysis of immunoblots from experiments shown in panel D ($n = 3$; $p > 0.05$). (F) Nuclear localization of wt-EGFR and EGFR-K37R in PAE cells assessed by subcellular fractionation. Na,K-ATPase (membrane marker) and histone H3 (nuclear marker) were blotted as fractionation controls. (G) Stability of wt-EGFR and EGFR-K37R investigated in PAE cells after 30 μg/mL cycloheximide treatment for 12 or 24 h. (H) Densitometric ratios of total EGFR after treatments shown in panel G, normalized to GAPDH ($n = 3$).

performed a preliminary investigation of whether SUMOylation of K37 might modulate the transactivation activity of nEGFR. We here chose to focus on two of the transcription targets of nEGFR: c-myc (*MYC*) and cyclin D1 (*CNND1*) genes. To evaluate protein expression, we transfected PAE cells with the mock, wt-EGFR, or EGFR-K37R construct. We could first confirm that both wt-EGFR and K37R are phosphorylated under basal conditions (Figure S4), followed by immunoblot-

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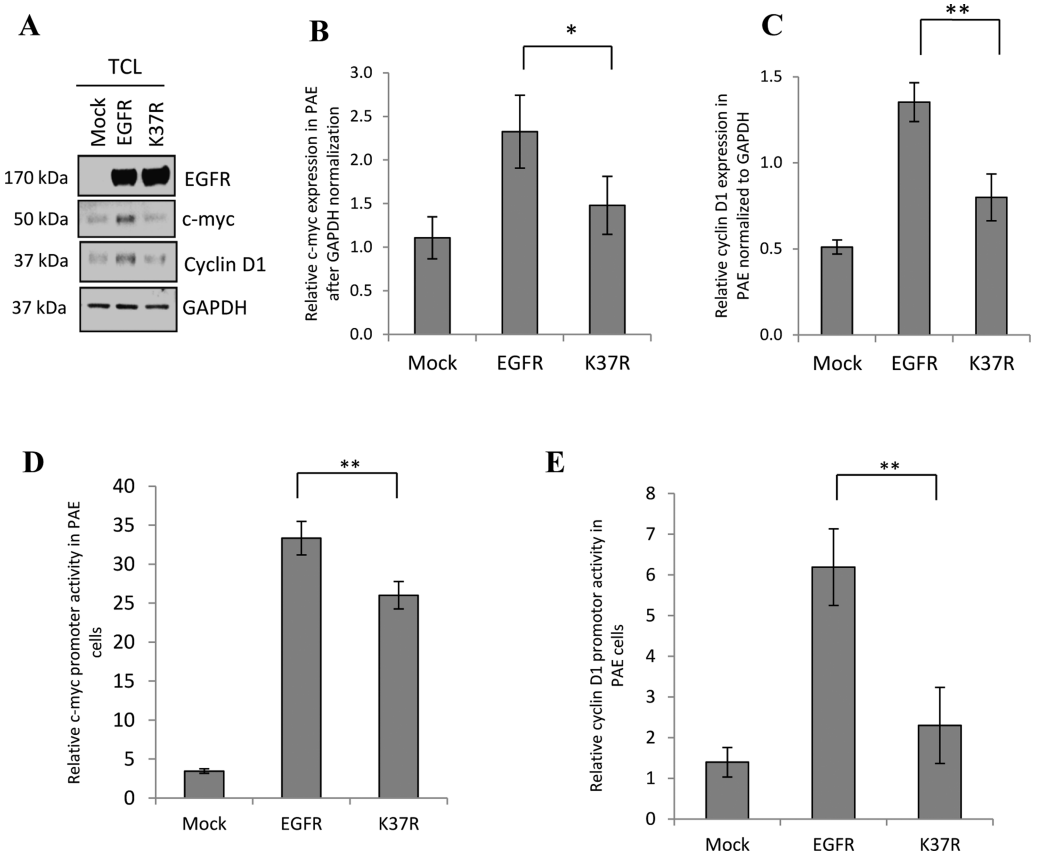

Figure 5. EGFR-K37R affects protein expression and transcriptional activity of *CNND1* and *MYC*. (A) Total cell lysates from PAE cells transfected with the mock, wt-EGFR, or EGFR-K37R construct were analyzed for protein expression of EGFR, c-myc, and cyclin D1. (B and C) Densitometric ratio of c-myc and cyclin D1 protein expression after normalization to GAPDH. Means and SD are shown (n = 3). (D and E) Cells described in panels A–C were cotransfected with *Renilla* luciferase (internal control) and either the *MYC* promoter or the *CCND1* promoter fused with luciferase. Data represent the average luciferase activity of raw data after normalization against *Renilla*. Means and SD are shown (n = 3).

ting against EGFR, c-myc, and cyclin D1 (Figure 5A). Both c-myc and cyclin D1 were significantly reduced in EGFR-K37R compared to wt-EGFR [43 and 54%, respectively (Figure 5B,C)]. To investigate if this change could be indeed due to alterations in EGFR-dependent transcriptional activity, we performed promoter luciferase gene reporter assays with mock-, wt-EGFR-, or EGFR-K37R-transfected PAE cells. For the c-myc promoter luciferase plasmid, only a partial sequence of the promoter was encoded, covering the STAT3 binding domain, while the cyclin D1 plasmid contained the complete promoter region. Results show that the *MYC* and *CNND1* promoter activities are significantly reduced in the K37R mutant compared to those in the wild-type receptor [22 and 63%, respectively (Figure 5D,E)].

DISCUSSION

To the best of our knowledge, this is the first study showing that EGFR is SUMOylated by both SUMO-1 and SUMO-2/3. Interestingly, essentially all of the SUMO-1-modified receptor is localized inside the cell nucleus in an endogenous setting, but by inducing a hyper-SUMOylation state through overexpression of Ubc9/SUMO-1, we can detect the EGFR–SUMO-1 complex outside the nucleus, as well. By abolishing all SUMOylated EGFR, by overexpression of SENP2, followed by cellular fractionation showing no decrease in nEGFR, we conclude that EGFR SUMOylation is not the main effector of the nuclear translocation of receptors. We also demonstrated that EGFR SUMOylation is mediated by EGF and peaks after ligand stimulation for 20–30 min.

Denatured IP showed multiple EGFR SUMOylation bands, suggesting that the receptor has multiple SUMO-1 molecules covalently attached at the same time. To identify the SUMOylation sites, we employed two alternative mass spectrometric strategies. However, because both MS approaches pointed to lysine 37 as the potentially SUMOylated site and K37 was the only lysine residue that passed the confidence threshold, we focused on exploring the functional importance of K37 SUMOylation.

We first showed that the K37R mutant can activate phosphorylation of Akt and Erk after EGF stimulation as strongly as wt-EGFR, suggesting that it does not impair ligand binding, although the interaction between EGF and EGFR might have been altered. We cannot exclude the possibility that modifications of the extracellular region will cause a conformational change in the intracellular region; thus, it could potentially affect downstream signaling through changes in, for example, binding of adapter molecules. Further, the stability and nuclear translocation were also unaffected in the K37R mutant compared to those in wt-EGFR. We cannot exclude the possibility that other SUMO-1 modification sites of EGFR are important for the stability. However, the intact translocation is in line with our results, suggesting that the receptor is mainly SUMOylated first after nuclear translocation has occurred, shown by fractionation IP as well as PLA. This result also

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correlates with the previous finding that it is the juxtamembrane region of the EGFR, containing a nuclear localization signal (NLS), that is required for its nuclear translocation,[41] while lysine 37 is situated in the extracellular domain of the receptor.

It has already been proven that the full length receptor translocates into the cell nucleus.[13,17] Wang et al. have further described that cell surface EGFR translocates from the nucleus, via the endoplasmic reticulum, to the inner nuclear membrane, where the full length receptor is further associated with sec61β, which causes the receptor to release from the lipid bilayer into the nucleus.[42] This mechanism would make it possible for SUMO E3 ligases situated within the cell nucleus, e.g., ligases belonging to the PIAS family, to SUMOylate the extracellular part of EGFR. However, to verify this hypothesis and determine which SUMO E3 ligase is necessary for EGFR SUMOylation, further studies need to be conducted.

As SUMOylated EGFR is localized in the cell nucleus, we also wanted to investigate whether this modification could affect nuclear EGFR-dependent transcriptional activity. Herein, we examined the expression of c-myc and cyclin D1 after overexpression of wt-EGFR and EGFR-K37R in EGFR deficient cells. The levels of both proteins are significantly reduced in cells transfected with the mutant. This finding was further supported by the promoter luciferase reporter assay showing that EGFR-K37R significantly reduces the promoter activity of both genes. These results, together with the unaffected downstream signaling of K37R observed in Figure 4D, indicate that SUMOylation affects nEGFR-mediated transcription, although we cannot exclude the possibility that other downstream effectors are affected by this mutant or that the observed phenotype of K37R is SUMO-independent by interfering with the binding of other modifications such as ubiquitylation and acetylation. The mechanism behind the observed effects on *MYC* and *CNND1* transcription needs to be studied further, but one possible hypothesis is that SUMOylated EGFR may stabilize the binding between STAT3 and RNA helicase A, transcription factors shown to associate with nEGFR and induce transcription of *MYC* and *CNND1*, respectively.[14,29] While this is just an initial study of SUMO-1 modification of EGFR, we believe that our findings may contribute to an improved understanding of the nuclear regulatory effects by EGFR.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00640.

EGFR−SUMO-2/3 interactions as determined by a PLA (Figure S1), mass spectra of the MALDI-TOF peptide mass fingerprint (Figure S2), nEGFR in H1299 after serum starvation followed by EGF stimulation at different time points (Figure S3), and EGFR phosphorylation of wt-EGFR and K37R transfected in PAE cells grown under basal conditions with serum (Figure S4) (PDF)

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Notes

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