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Nuclear EGFR shuttling induced by ionizing radiation is regulated by phosphorylation at residue Thr654

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ABSTRACT

Nuclear localisation of EGFR is associated with treatment resistance of tumor cells. The aim of this study was to identify molecular targets to block nuclear shuttling of EGFR. Mutation of Thr654, located within the putative EGFR NLS demonstrated that phosphorylation of this residue is essential for nuclear EGFR shuttling following irradiation. Deletion of Thr654 blocked nuclear transport of EGFR, whereas mutation to Glu increased shuttling. Treatment with a peptide, corresponding to the phosphorylated NLS, abolished nuclear EGFR transport and reduced radiation-induced activation of DNA-PK, essential for DNA-repair. In accordance with that, lack of nuclear EGFR increased residual DNA damage in tumor cells and reduced cellular survival following irradiation. Blockage of nuclear EGFR shuttling may be a new strategy to fight treatment resistance.

Structured summary:
MINT-7987956: *Karyopherin alpha* (uniprotkb:P52294) *physically interacts* (MI:0915) with *EGFR* (uniprotkb:P00533) by *anti bait coimmunoprecipitation* (MI:0006)

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1. Introduction

Many human tumors are characterized by an over-expression of epidermal growth factor receptor (EGFR), a protein that promotes resistance to chemo- and radiotherapy [1–4]. EGFR can be activated through phosphorylation of specific amino acid residues in response to ligand binding (EGF, TGF alpha and amphiregulin) [5,6] as well as exposure to a variety of unspecific stimuli, including ionizing radiation [Schmidt-Ullrich, 1997 #195], UV radiation [7], hypoxia [8], hyperthermia [9], oxidative stress [10] and trans-activation by G-protein coupled receptors [11]. Both ligand-dependent and ligand-independent phosphorylation of EGFR result in receptor internalization [12] and intracellular signaling [1,4,13–16].

Ionizing radiation also induces EGFR phosphorylation and internalization [17] which is not only linked to activation of EGFR-dependent cytosolic signalling (PI3K, MAPK) [18], but also to nuclear EGFR transport [17,19]. To pass through the nuclear pore complex, EGFR must associate with the karyopherin nuclear import complex [19]. Many proteins are imported via karyopherin β, often using karyopherin α as an adaptor. A prerequisite for karyopherin-binding is the presence of a nuclear localization site (NLS) within the cargo protein. Classical NLS's contain one or two clusters of basic residues [20]. Lin et al. [21] identified a putative NLS within EGFR sequence. We were the first to report, that EGFR is found in complex with karyopherin α and RAN-GTP [14] after exposure of cells to ionizing radiation. Phosphorylation of EGFR at residue T654, which is located within this putative EGFR NLS, was reported to be associated with radiation-induced nuclear EGFR transport. Furthermore, we identified PKCε as the kinase responsible for this modification following irradiation [22]. As already reported earlier [23] EGFR T654 phosphorylation blocked Cbl-induced ubiquitination and lysosomal degradation of EGFR following ligand activation leading to EGFR stabilization.

High levels of EGFR have been detected in the nuclei of many tumors, including adrenocorticoid, breast, bladder, skin, thyroid and mouth [21,24–27]. Nuclear EGFR positively correlates with Ki-67 expression, an indicator of proliferation, suggesting that nuclear EGFR may activate transcription [26]. Indeed, nuclear EGFR targets the promoters of cyclin D1, iNOS and B-Myb [21,28–31],

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the expression of which is necessary for cell cycle progression and proliferation.

Moreover, Bandyopadhyay et al. [32] reported, that nuclear EGFR undergoes physical interaction with DNA-dependent kinase (DNA-PK), which is a major enzyme of non-homologous end-joining DNA-double strand break repair. Nuclear EGFR is associated with phosphorylation of DNA-PK at residue T2609, an indicator of DNA-PK activity during non-homologous end-joining DNA repair [14]. Blockage of nuclear EGFR transport by the antibody Erbitux decreased DNA-PK activity, increased residual DNA damage and reduced survival after radiation exposure of the human lung carcinoma cell line A549 [14]. These observations suggest a crucial role for nuclear EGFR in regulation of DNA repair following treatment with genotoxic substances and identify blockage of nuclear EGFR shuttling as a new molecular intervention strategy to fight treatment resistance of tumors.

2. Materials and methods

2.1. Cell culture, irradiation and colony formation assay

Experiments were performed using the human bronchial carcinoma cell line A549 and Chinese hamster ovary CHO-K1 cells from ATCC (Rockville, MD, USA). Cells were irradiated with 200-kV photons (Gulmay RS 225, dose rate 1 Gy/min) at 37 °C. For colony formation assay, cells were grown to confluence, treated as indicated, and irradiated. After 6 h, cells were typsinized and seeded at a density of 500 cells in 78 cm^2 plates. After 10 days, colonies were fixed, stained, and counted. The transport inhibitory T654-peptide (Ac-RKRT(PO3H2)LRRLK-fluorescein) and the corresponding control peptide (KKALRRQEAVNAL-fluorescein) were synthesized by Genaxxon Bioscience (Ulm, Germany). Cells were incubated with both peptides for 16 h at a concentration of 5 μM.

2.2. Transfection

Transfection of the CHO cells was performed with electroporation (Amaxa, Walkersville, MD, USA) according to the manufacturer's instructions. We used the EGFR full ORF expression clone (RZPDo839G0149, Deutsches Ressourcenzentrum für Genomforschung rzpd, Berlin Germany) cloned in vector pDEST26. With the help of specific PCR-primers and the site-specific mutation kit (Stratagene, La Jolla, CA, USA), the residue T654 was deleted (5′-CCACATCGTTCGGAA-GCGCCTGCGGAGG-3′) or substituted by Glu (5′-CGCCACATCGTTCGGAAGCG-CGAGCTGCGGAGGCTG-3′). Vectors coding for either wild type (wt) or mutated EGFR were transfected into CHO K1 cells. Selection of transfected clones was performed with neomycin (200 μg/ml). Stable clones were isolated by limited dilution cloning, and success of transfection was proven by direct sequencing of the region coding for EGFR from isolated vectors.

2.3. Subcellular fractionation

Cytoplasmic and nuclear extracts were prepared according to the instructions of the NE-PER® nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL, USA).

2.4. Confocal microscopy

CHO cells were cultivated on CultureSlides (Becton Dickinson, Franklin Lakes, NJ, USA), irradiated with 4 Gy, and after 10 min, fixed with periodate-lysine-paraformaldehyde (PLP). For immunofluorescence analysis, cells were incubated with anti-EGFR (BD Bioscience, clone 13) (1:20) overnight at 4 °C. Bound antibodies were visualized by incubation with a 1:500 dilution of a Cy3-donkey anti–mouse serum (Dianova, Hamburg, Germany) for 1 h. Nuclei were stained with YO-PRO (Molecular Probes, Leiden, The

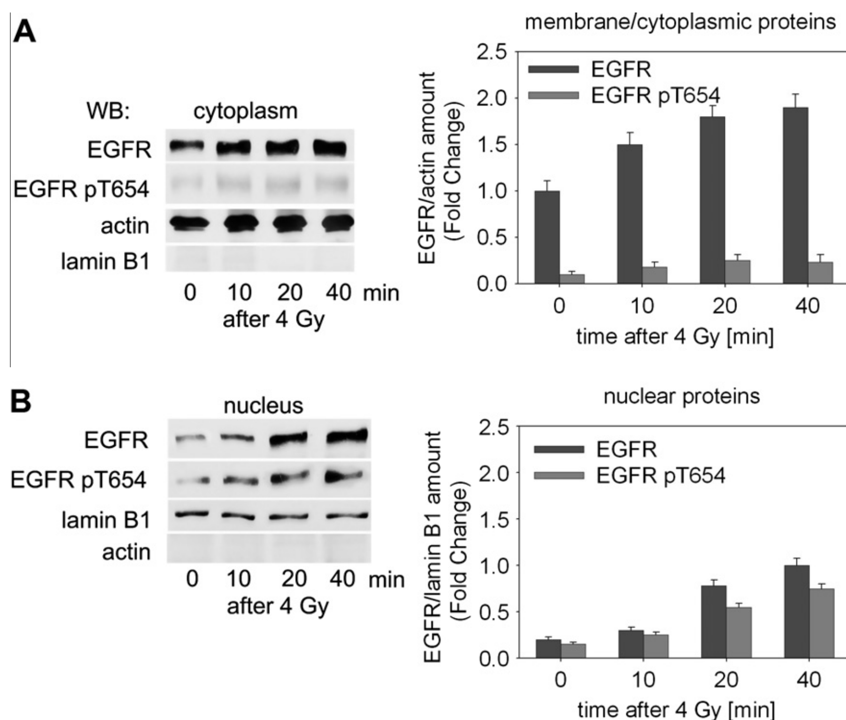

Fig. 1. Radiation-induced phosphorylation of EGFR at T654 within the cytosolic (A) and nuclear protein fraction (B). Confluent A549 cells were irradiated. At the time points given cells were lyzed, and cytosolic and nuclear proteins were isolated. Following protein separation, proteins were transferred to nitrocellulose. EGFR and EGFR phosphorylated at T654 were quantified with specific antibodies. Lamin B1 and actin were detected as loading controls. Protein quantification was performed by densitometry. The amount of protein present was normalized either with actin or lamin B1. Bars represent fold change relative to levels in sham-irradiated, untreated controls. Data are mean ± S.E.M., $n = 3$.

Netherlands). The nuclei were analyzed with a confocal laser scanning microscope (Leica TCS SP, Leica Microsystems, Bensheim, Germany).

2.5. Western blot analysis and immune-precipitation

After irradiation, cells were lysed, and proteins were resolved by SDS–PAGE. Western blotting was performed according to standard procedures. The primary antibodies were diluted as follows: anti-EGFR (BD Transduction Laboratories, clone 13) 1:1000; anti EGFR pT654 (nanotools, clone 3F2) 1:1000; anti-DNA-PK (PharMingen, clone 4F10C5) 1:500; anti-DNA-PK pT2609 (Rockland) 1:1000; anti-lamin B1 (Biozol, clone ZL-5) 1:1000; anti-His tag (Biozol, clone His.H8) 1:1000, anti-karyopherin α (BD Transduction Laboratories, clone 2) 1:1000). Quantification of binding was achieved by incubation with a secondary peroxidase-conjugated antibody with the ECL system (Amersham).

EGFR was immune-precipitated from cytosolic and nuclear protein fractions prepared from 20×10^6 cells with EGFR antibody clone 13 (BD Transduction Laboratories). Immune-precipitation was performed as described previously [14].

2.6. Quantification of γH_2AX-foci formation

Confluent cells cultured on CultureSlides (Becton Dickinson) were irradiated and fixed with 70% ice-cold ethanol 24 h after irradiation. For immune-fluorescence analysis, cells were incubated with γH_2AX antibody (Upstate, clone JBW301) (1:500) at room temperature for 2 h. Positive foci were visualized by incubation with a 1:500 dilution of Alexa488-labelled goat anti-mouse serum (Molecular Probes) for 30 min. CultureSlides were mounted in Vectashield/DAPI (Vector Laboratories). For each data point, 300–500 nuclei were evaluated.

3. Results

Radiation exposure of A549 cells markedly increased the amount of EGFR in the cytosolic protein fraction, but cytosolic EGFR was only moderately phosphorylated at Thr residue no. 654 following radiation (Fig. 1A). In contrast, radiation exposure increased EGFR within the nuclear fraction and this increase was correlated with a pronounced increase in EGFR phosphorylation at residue T654 (Fig. 1B).

To elucidate the role of T654 phosphorylation for nuclear EGFR transport, CHO cells, characterized by a low level expression of endogenous EGFR, were stably transfected with an expression vector coding for an EGFR either with a deletion of T654 or a mutation of T654 to Glu – which mimicks a permanent phosphorylation at this site – or a wt. EGFR. All three cell lines expressed the constructs and responded with EGFR stabilization upon irradiation (Fig. 2A) (His-tag-EGFR). In CHO cells, expressing wt. EGFR an efficient nuclear translocation of EGFR following radiation exposure

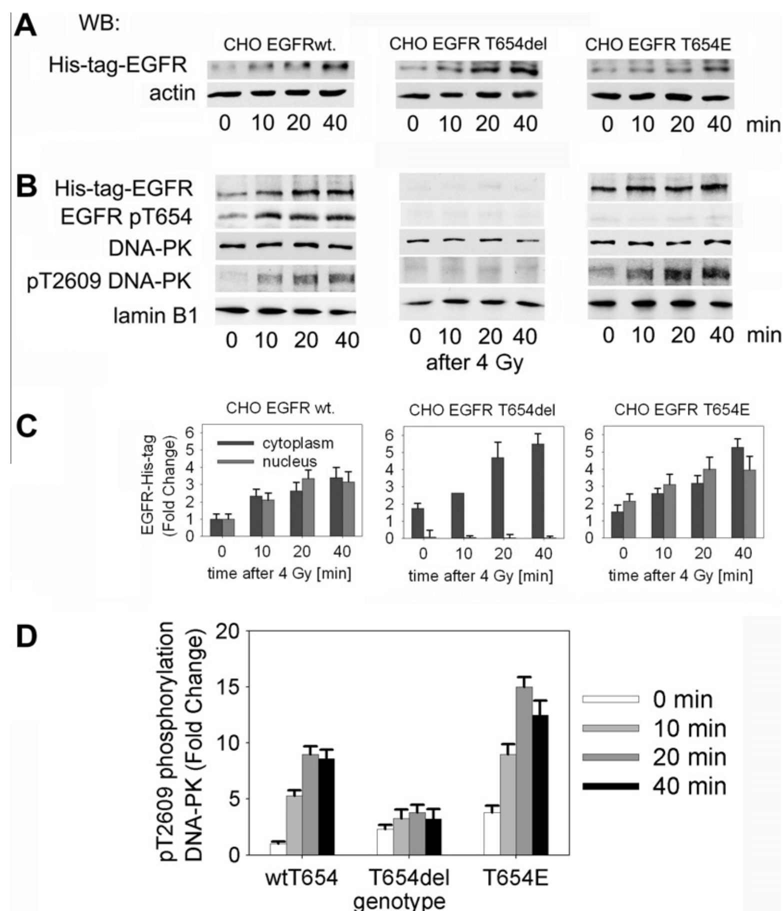

Fig. 2. Cytosolic (A) and nuclear EGFR protein (B) in cells expressing EGFR mutated at residue T654. Expression vectors coding for wt. EGFR or mutated at T654 were transferred to CHO cells. Stable expressions of wt. and mt. EGFR were followed by detection of a co-expressed His-tag. The amino acid threonine (T) in position No. 654 was deleted (del), or substituted by glutamic acid (Glu). CHO cells were irradiated, and at the time points given, cytosolic and nuclear proteins were isolated and His-tagged EGFR was detected after western blotting. Quantification was performed by densitometry (C). Relative DNA-PK phosphorylation at residue T2609 was shown in panel (D). The amount of proteins present was normalized either with actin, lamin B1, or DNA-PK. Bars represent fold change relative to levels in sham-irradiated, untreated controls. Data are mean ± S.E.M., $n = 3$.

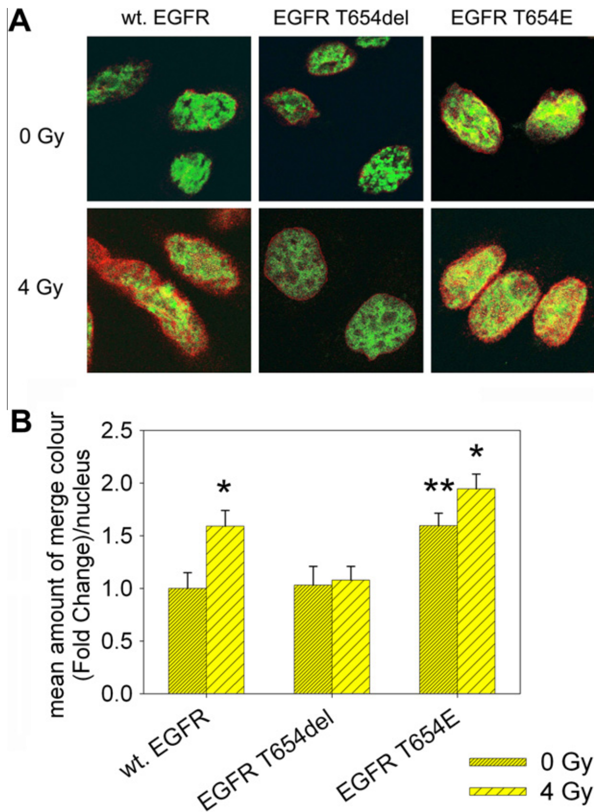

Fig. 3. Expression vectors coding for EGFR protein mutated at T654 were stably transferred to CHO cells. Radiation induced nuclear EGFR transport was visualized by confocal microscopy and immune-staining (DNA was stained by YOPRO = green, EGFR in red (Cy3) (A). Colocalisation was quantified by detection of merge colour yellow with help of Leica TCS-software (B). For each bar 50 nuclei were evaluated. Data are mean expression ± S.E.M., $^{*}P < 0.05$ compared to not irradiated control, $^{**}P < 0.05$ compared to not irradiated control.

could be demonstrated (Fig. 2B). As described earlier [14] nuclear EGFR transport was associated with increased DNA-PK phosphorylation at residue T2609 (Fig. 2B). However, in cells expressing an EGFR missing T654, both radiation-induced nuclear EGFR translocation and DNA-PK phosphorylation at T2609 were abolished (Fig. 2B–D). Interestingly, mutation of T654 to Glu promoted basal nuclear EGFR levels and radiation-associated nuclear import markedly (Fig. 2B). Moreover, in these cells, radiation-induced EGFR translocation was associated with DNA-PK phosphorylation at T2609.

These observations were confirmed by confocal microscopy. In cells expressing wt EGFR, radiation exposure induced EGFR translocation to the nucleus (Fig. 3). EGFR (in red) disappeared from its peri-nuclear location and was detected within the nucleus (DNA in green). Physical co-localization of EGFR with DNA was visualized by the appearance of yellow spots (merge colour) within the nucleus. In contrast, in cells expressing EGFR missing T654, radiation-induced EGFR transport could not be detected (Fig. 3A and B). However, in cells carrying the T654 to Glu mutation, EGFR was detectable within the nucleus even without radiation exposure (Fig. 3A and B).

Earlier data [14] indicated, that the karyopherin transport system is involved in nuclear EGFR transport following irradiation. Therefore, we tested complex formation between endogenous karyopherin α and EGFR in CHO cells carrying the EGFR T654 deletion or T654E mutation with help of immune-precipitation experiments. Cells expressing wt. EGFR clearly showed complex formation between EGFR and karyopherin α following irradiation within cytosol (Fig. 4A). Under these conditions T654 phosphorylated EGFR was detected in complex with karyopherin α. However, in cells expressing EGFR with the T654 deletion complex formation was markedly reduced. Complex formation of EGFR and karyopherin α was apparent in cells carrying the T654E mutation even without radiation exposure by ionizing radiation. Moreover, the corresponding pattern was detected in the nuclear protein fraction also (Fig. 4B). Deletion of Thr 654 within the EGFR sequence reduced EGFR/karyopherin α complex formation significantly (Fig. 4A and B). In contrast, in cells carrying the EGFR T654E mutation complex formation was observed constitutively.

To further prove the crucial role of phosphorylated T654 in radiation-induced nuclear EGFR transport, we incubated A549 cells with a peptide, corresponding to the putative endogenous EGFR NLS. A 16 h pre-treatment with this peptide phosphorylated at T654 markedly blocked nuclear EGFR shuttling following irradiation (Fig. 5A). Likewise, radiation-induced phosphorylation at T654 was inhibited similarly and this inhibition was associated with a marked decrease of radiation-induced phosphorylation of

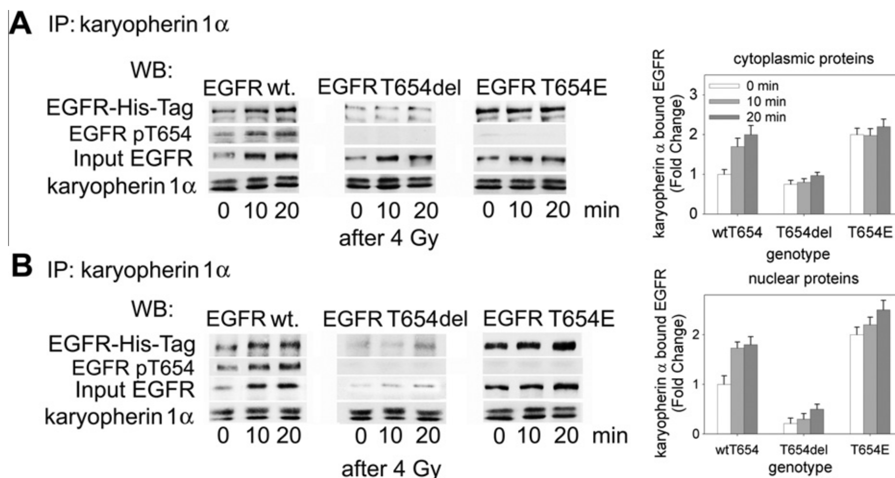

Fig. 4. EGFR binding to karyopherin a in cytoplasm (A) and nucleus (B). CHO cells expressing wt. EGFR, EGFR T654del or EGFR T654E were irradiated with 4 Gy. At the time points given, cells were lyzed and an immune-precipitation of karyopherin α was performed. Karyopherin-bound EGFR in the cytosolic (A) and nuclear fraction (B) was detected after PAGE and western blotting by help of His-tag. In addition, EGFR and EGFR phosphorylated at T654 were detected in the input protein fraction. Protein quantification was performed by densitometry. The amount of protein present was normalized with karyopherin a. Bars represent fold change relative to levels in sham-irradiated, wt. EGFR expressing, untreated controls. Data are mean ± S.E.M., $n = 3$.

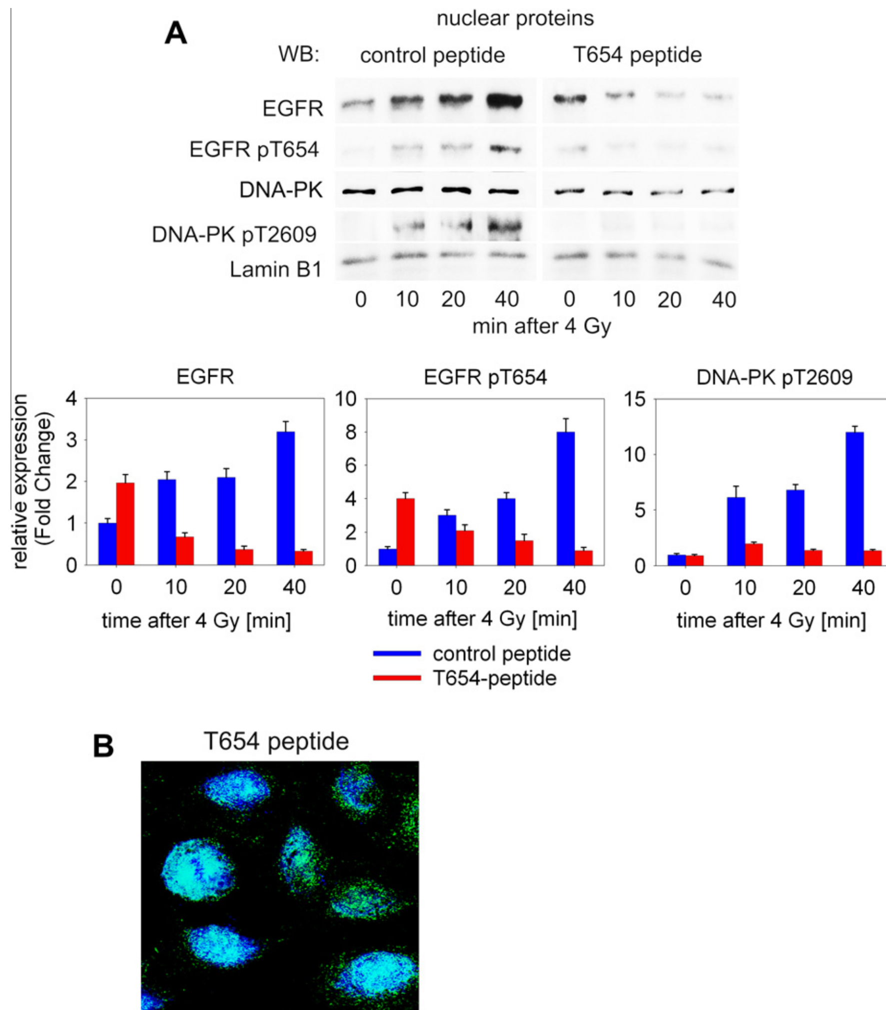

Fig. 5. Inhibition of radiation-induced nuclear EGFR translocation and phosphorylation of DNA-PK by means of phosphorylated T654-peptide. A549 cells were incubated with the T654-peptide corresponding to the putative EGFR NLS (Ac-RKRT(PO3H2)LRRLK-fluorescein) and the control peptide (KKALRRQEAVNAL-fluorescein) for 16 h. Cells were irradiated, and nuclear protein extracts were isolated at the time points given. Lamin B1 and DNA-PK were detected as loading controls (A). Protein quantification was performed by densitometry. The amount of protein present was normalized either with lamin B1 or DNA-PK. Bars represent fold change relative to levels in sham-irradiated, untreated controls. Data are mean ± S.E.M., $n = 3$. Cellular localization of the peptides was visualized by detection of T-654-fluorescein peptide (green) and nuclear dye YOPRO-1 (blue) (B).

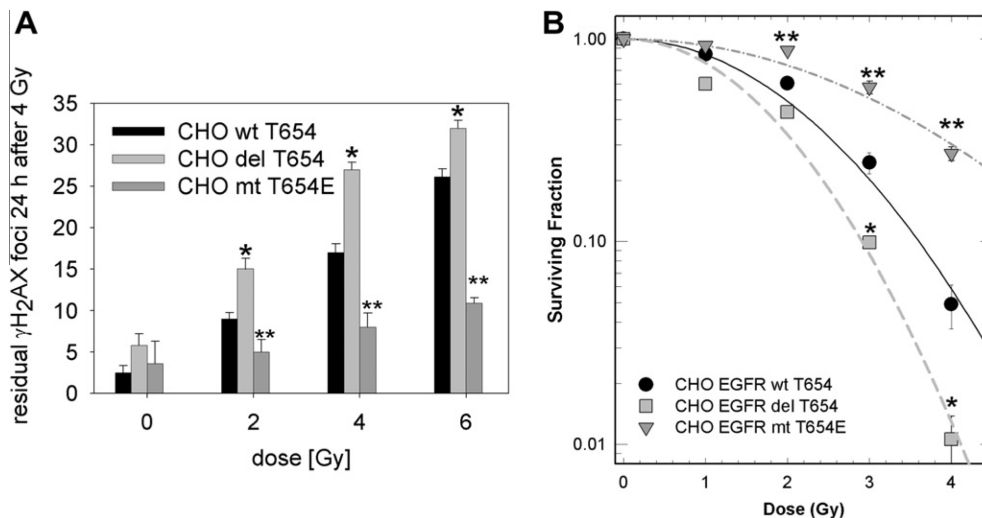

Fig. 6. Effect of EGFR T654 mutations on DNA-repair and cell survival. (A) Effect upon DNA-damage repair. Confluent CHO cells expressing either wt. EGFR, EGFR T654del or EGFR T654E where irradiated with different radiation doses. After 24 h, cells were fixed and the amount of residual damage was quantified by determining the amount of gH2AX-positive nuclear foci. For each data point 300–500 nuclei were evaluated. (B) Confluent CHO cells expressing either wt. EGFR or T654 mt. EGFR were irradiated with different radiation doses. After 6 h of DNA repair time, cells were plated for colony formation assay. Surviving curves were based on at least three independent experiments. $^{*}P < 0.05$ compared to CHO wt. T654, $^{**}P < 0.05$ compared to CHO wt. T654.

DNA-PK. Interestingly, radiation-independent basal nuclear EGFR transport was still increased. However, the radiation-independent increase in nuclear EGFR in response to treatment with the T654 peptide had no effect on DNA-PK phosphorylation (Fig. 5A). To prove nuclear localisation of phosphorylated T654 peptide, cells were incubated either with T654 peptide or with control peptide both labelled with FITC. Both peptides were detected intracellular after 16 h labelling time, but only the phosphorylated T654 peptide accumulated within the nucleus (Fig. 5B).

To investigate whether blockage of nuclear EGFR transport affects DNA repair following irradiation, we quantified residual DNA-double strand breaks in CHO cells 24 h after radiation exposure. At all radiation doses, cells expressing EGFR with the T654 deletion presented an increased amount of residual damage compared to cells expressing the wt. EGFR (Fig. 6A). However, cells expressing EGFR with the T654E mutation demonstrated a reduced amount of residual damage (Fig. 6A). This effect was prominent at all radiation doses, and the residual DNA damage was reduced by about 50%. These results were in agreement with data obtained from colony formation assays testing the clonogenic survival after irradiation (Fig. 6B). Cells expressing an EGFR with a T654E mutation were highly radioresistant as compared to cells expressing wt. EGFR (Fig. 6B). In contrast, cells expressing EGFR with T654 deletion were markedly radiosensitized (Fig. 6B).

4. Discussion

Recent data reported localization of the membrane receptor EGFR within the cell nucleus [14,21]. Although, the functional importance of this observation is not yet resolved, several clinical reports suggest that nuclear EGFR mediates resistance to cancer treatment regimes, leading to a poor clinical outcome [26,31,33,34].

Based on the observation that radiation exposure mimics phosphorylation of several EGFR amino acid residues [35], we hypothesized that phosphorylation of EGFR regulates its translocation into the nucleus. Although cytosolic EGFR was phosphorylated at several residues following irradiation, i.e., Y845 [17], Y1173 [36], none of these tyrosine phosphorylations could be correlated with nuclear EGFR transport (data not shown). However, after radiation exposure, nuclear EGFR was markedly phosphorylated at residue T654. Recently, studies have shown, that PKC ε is activated by irradiation, and was identified to be responsible for EGFR T654 phosphorylation [22]. T654 phosphorylation is involved in regulation of kinase activity [37] and blockage of ubiquitin dependent EGFR degradation [23]. Furthermore, the preferential detection of T654 phosphorylation of EGFR within the nuclear fraction also suggests a functional role of this phosphorylation in the nuclear translocation mechanism of EGFR induced by irradiation. Previously, we have shown, that nuclear EGFR transport is mediated via the karyopherin transport system [14], which includes binding of karyopherin to a specific nuclear localization site [38]. Interestingly, the residue T654 is located within the putative nuclear localization site (NLS) of the EGFR as described by Lin et al. [21]. The structure of NLS's is characterized by the prevalence of amino acids with basic side chains [38]. Phosphorylation of a residue within this site would dramatically change the net charge and may be of functional relevance for binding to karyopherin α. Indeed, phosphorylations have been shown to regulate activity of some NLS's [39]. Herein, we demonstrate, that preventing phosphorylation of the T654 residue by knockout abolished radiation-induced nuclear EGFR transport. This result fits with the observation that EGFR phosphorylation at T654 could preferentially be detected within the nucleus. It can be assumed, that T654 phosphorylation triggers nuclear transport of EGFR. This idea is further supported by data obtained from the T654E substitution within the EGFR sequence. This mutation added a negative charge to the basic NLS sequence, mimicking permanent phosphorylation. Consequently, EGFR was constitutively shuttling into the nucleus, even without irradiation. These observations were confirmed by confocal microscopy, which also clearly demonstrated, that EGFR shuttling to the nucleus required T654 phosphorylation.

To determine the functional consequence of phosphorylation at T654 on the transport process, we analyzed the formation of the EGFR/karyopherin α complex. Immune-precipitation experiments using karyopherin α suggest, that phosphorylation of T654 in the cytosolic fraction is essential for binding of karyopherin α. Deletion of T654 strongly reduced complex formation between EGFR and karyopherin α, despite sufficient EGFR protein expression in cytoplasm. Consequently, the T654 deletion also reduced the amount of EGFR transported to the nucleus. Interestingly, the data obtained with the T654E mutation indicate, that a negative charge in position no. 654 is sufficient to support EGFR/karyopherin α complex formation.

The use of the phosphorylated T654-peptide (corresponding to the EGFR NLS), gave further evidence to the crucial role of the T654 site in regulating EGFR transport into the nucleus. Our data suggest, that in irradiated cells the phosphorylated NLS-peptide competes with EGFR phosphorylated at T654 for binding to karyopherin α and impairs nuclear EGFR transport. Yet, increased amount of nuclear EGFR in non-irradiated cells after incubation with phosphorylated T654-peptide alone cannot be explained and does require further evaluation. However, possible explanations would be that either the T654-peptide induced a compensatory effect during the 16 h incubation time, which led to an EGFR transport, or that basal amount of nuclear EGFR was regulated independently of NLS and karyopherin α. Nevertheless, the effect of the phosphorylated T654-peptide on radiation-induced EGFR nuclear shuttling is very clear and is associated with a reduced phosphorylation of DNA-PK at residue T2609. This links nuclear EGFR with DNA-double strand break repair and is confirmed by previous results [14]. Following DNA damage, DNA-PK T2609 phosphorylation in concert with additional phosphorylations [40,41] lead to conformal structural changes enabling Artemis ligase access to the open ends of double strand breaks caped by the DNA-repair complex, a prerequisite for successful DNA-repair [40].

The data presented herein demonstrated, that blockage of nuclear transport of EGFR by deletion of T654 in the EGFR-NLS, led to increased residual damage following radiation exposure. Moreover, the observation, that nuclear EGFR is involved in regulation of DNA-PK function is consistent with the observed increase in DNA damage of cells with impaired EGFR nuclear import. Inhibition of DNA-repair in the stationary phase, in which the majority of cells is arrested in G0- or G1-cell cycle phase, suggests an effect upon non-homologous end-joining DNA-repair (NHEJ), the predominant repair mechanism for DNA-double strand breaks in G1 [42]. As reported by Rothkamm et al. [42], the amount of residual damage 24 h after irradiation correlates significantly with clonogenic cell survival tested in a colony formation assay. Surprisingly, in cells carrying the T654E mutation, constitutive translocation of EGFR into the nucleus stimulated DNA repair and markedly increased radioresistance. One possible explanation is that constitutive EGFR nuclear shuttling causes the repair complex to be in a pre-activated state, enabling more efficient and faster DNA repair after radiation exposure.

5. Conclusions

Taken together, our results demonstrate the crucial role of T654 phosphorylation within the NLS of EGFR in radiation-induced nuclear accumulation of EGFR via a karyopherin-driven process. Furthermore, with respect to modulation of nuclear EGFR and its consequences on survival of irradiated cells, modifying nuclear

transport may become a new clinical strategy to overcome treatment resistance of tumors.

Conflict of interest statement

We declare that we have no conflict of interest.

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