compelling theory to explain CUP is that the primary cancer is microscopic and may disappear because of marked apoptosis after seeding metastases that are able to proliferate into more significant tumors in different tissues [27]. As a high metastasis potential and vulnerability to apoptosis would explain the properties of CUP well, we first searched for genes related to metastasis and apoptosis among all the genes that were up-regulated by more than 2.5-fold in the CUP samples ($M_{CUP}(2.5)$ dataset).

Of the 14 up-regulated genes that were found (*S100A4, PRG1, S100A6, GSTP1, EIF5A, LGALS1, S100A11, PRKDC, VIM, CST3, TIMP1, YWHAZ, NEDD8, STK17A*), three (*S100A4, S100A6, S100A11*) belong to a group of S100 proteins involved in the Ca^{2+} signaling network and regulate a variety of intracellular activities including cell growth and motility [28]. The expressions of these genes are observed in several epithelial tumors and have been linked to metastasis [29,30]. *S100A4*, together with *VIM*, has also been used as an EMT marker [31]. The overexpression of *EIF5A* induces the EMT, thereby promoting the tumor metastasis of colorectal and hepatocellular carcinoma [32]. Serglycin, a gene product of *PRG1*, is a proteoglycan that has been functionally identified as a significant regulator of metastasis in nasopharyngeal carcinoma (NPC) [33]. The elevated expression of Serglycin in NPC cells can mediate the level of vimentin (*VIM*) expression, which is not only a marker of the EMT, but also has an important role in the regulation of cellular migration [31,34]. Lewis lung carcinoma cells in mice show metastasis to the lung when the cells express Galectin-1 (Gal-1), a large carbohydrate-binding protein encoded by *LGALS1*, suggesting novel targeting strategies for Gal-1 in cancer [35].

Both metastatic cells and drug-resistant cells have similar gene expression patterns of survival-related molecules, suggesting that metastatic cancer may be difficult to treat because of resistance to anticancer drugs. DNA-dependent protein kinase (DNA-PK), a gene product of *PRKDC*, is one of the proteins up-regulated in several metastatic and drug-resistant cancer cells [36]. Because the up-regulation of DNA-PK was observed in the CUP patients in our cohort, who had never been treated with chemotherapy, DNA-PK may indicate essential resistance, rather than acquired resistance, to chemotherapy. GSTP1 has also been postulated in several cancer types to enhance the metastatic potential and the development of resistance to drugs that induce reactive oxygen species (ROS), such as paclitaxel and cisplatin [37,38]. Other genes up-regulated in CUP also reveal a significant role in chemoresistance and may be linked to the metastatic potential. Breast cancer cells overexpressing TIMP-1, a well-known inhibitor of matrix metalloproteinase, exhibit a reduced sensitivity to the chemotherapeutic drugs paclitaxel and epirubicin through the activation of transcription factor NF-κB [39]. The knocked-down expression of 14-3-3 ζ, a gene product of *YWHAZ*, sensitizes head and neck cancer cells to chemotherapy [40]. A small molecule inhibitor of NEDD8 activating enzyme (NAE) may be active against tumors that are resistant to other chemotherapeutic agents [41].

Unlike the hitherto described genes, cystatin C (*CST-3*) and STK17A function as direct pro-apoptotic factors by antagonizing TGF-β signaling and by modulating ROS, respectively. Cystatin C has been shown to interact with the TGF-β type II receptor, thereby preventing TGF-β binding and subsequent EMT induction [42]. TGF-β has been accepted as a main initiator of EMT; however, NF-κB was recently found to promote EMT in some cells that are unresponsive to TGF-β because they lack functional SMAD4, representing an alternative pathway leading to EMT that can replace TGF-β signaling [43]. NF-κB signaling may predominately induce EMT in CUP. Both TIMP-1, which can activate NF-κB, and vimentin, which is activated by NF-κB, were among the genes (proteins) that were up-regulated in CUP as described above, making this hypothesis more likely [39,43]. STK17A is up-regulated in response to oxidative stress in a p53-dependent manner [44]. Since STK17A is known as a positive regulator of the apoptotic pathway and its expression level in colorectal carcinomas is enhanced in lesions with lymph node metastasis, the apoptotic process could be involved in the node metastasis of carcinomas, including CUP [45].

Of the 15 down-regulated genes in the $M_{CUP}(2.5)$ dataset, *CD24*, *KRAS* and *DICER1* are of particular interest. CD24 is the most widely used marker, together with CD44, for identifying tumor-initiating cells in breast carcinomas. $CD44^{+}/CD24^{-/low}$ breast cancer cells have the ability to metastasize, since the enrichment of these stem-like cells is significantly observed in patients with positive lymph nodes [46]. A subset of kras mutant cancer cells exhibit "kras addiction" and have a differentiated epithelial phenotype. The induction of EMT has been shown to convert kras-dependent cancer cells to kras-independent cells, which do not require the continued expression of kras [47]. *Dicer1* functions as a haploinsufficient tumor suppressor gene [48]. Frequent loss of one allele of *Dicer1* has been observed in several different tumor types causing a global reduction of steady-state micro RNA levels that could be functionally suppressive to the oncogenesis and metastasis of CUP.

The increased expression of several ribosomal proteins was found in CUP. Whether these changes in expression are causally related to the generation of CUP is unknown. In some cases, the overexpression of ribosomal proteins, including RPL5, RPL11, RPL23 and RPS7 has been shown to suppress tumorigenesis [49,50]. These proteins activate p53 by binding to MDM2 and inhibiting MDM2-mediated p53 ubiquitination and degradation in response to nucleolar stress (also called ribosomal stress). RPL11 and RPS7 were recently shown to be required for p53 activation induced by DNA-damaging agents [51], suggesting that these ribosomal proteins may play a crucial role in p53 activation in response to diverse stressors. Furthermore, neddylation, the process by which the ubiquitin-like protein NEDD8 is conjugated to its target, is essential for RPL11's role in the mediation of p53 signaling [49]. Interestingly, these two ribosomal proteins and NEDD8 were included in our $M_{CUP}(2.5)$ dataset. The tumor suppressor function performed by these proteins may be related to the vulnerability to apoptosis that CUP (at the primary site) exhibits as one of its properties.

For functional analyses of the identified genes, overexpression or knockdown experiments using appropriate cell lines would be plausible to pursue if the gene of interest confers change in growth or in metastatic ability to the cells. The metastatic process can be evaluated *in vitro* by monitoring cell invasion through Matrigel and adhesion of cells to plates, etc. Synthetic inhibitors specific to Gal-1, DNA-PK and 14-3-3 ζ have been developed [52–54]. Thus, it will be intriguing to investigate the effect of these inhibitors on the cells overexpressing the respective gene *in vitro* or *in vivo*, which may lead to targeted therapy for CUP.

To our surprise, the gene expression profile (GEF) of CUP closely resembled that of lung adenocarcinoma (LAC), which may simply reflect the relatively high metastatic potential of LAC. In a study using ^{18}F-fluoro-2-deoxyglucose positron emission tomography (FDG-PET), the most commonly detected location of the primary tumor in patients with CUP was the lung [55]. In CUP, the primary cancer and its metastasis (-ses) behave very differently in respect to proliferation, leading to the assumption that the molecular profiles of CUP specimens from the two sites would differ. We are unable to compare these differences because the

primary cancer is unidentifiable. A differential gene expression analysis using primary and metastatic tumor tissues from advanced lung cancer patients may provide some clues to this question.

In conclusion, we identified several genes that were up-regulated in CUP and that may contribute to the acquisition of a metastatic phenotype as well as resistance to anticancer drugs in many cases. Proapoptotic factors were also identified. The combinational effects of the multiple functions of genes that are highly expressed in CUP could be involved in regulating CUP behaviors, such as apoptosis and metastasis. Immunohistochemical-based or PCR-based validation of the candidate genes is needed to refine the molecular classification of CUP.

Supporting Information

Figure S1 Heatmap constructed as in Figure 1 but excluding the *VAPA* gene.
(TIF)

Figure S2 Cluster dendrogram for each cancer type. Clustering analysis was done using the Ward method and 77 ribosomal protein genes.
(TIF)

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Author Contributions

Conceived and designed the experiments: IK TK YK K. Nakagawa K. Nishio. Performed the experiments: YF YK KS. Analyzed the data: IK YF TA. Contributed reagents/materials/analysis tools: TK K. Matsumoto MT KT YT NY AT NM H. Mukai H. Minami NC YY K. Miwa Shin Takahashi Shunji Takahashi. Wrote the paper: IK YF K. Nishio.

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Sorting nexin 2-mediated membrane trafficking of c-Met contributes to sensitivity of molecular-targeted drugs

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The sorting nexin (SNX) family is a diverse group of cytoplasmic and membrane-associated proteins that are involved in membrane-trafficking steps within the endocytotic network. SNX1 and SNX2 are components of the mammalian retromer complex and they also play critical roles in the membrane trafficking of growth factor receptors including epidermal growth factor receptor (EGFR) and c-Met. The human lung cancer cell lines, which harbor activating mutations in the kinase domain of *EGFR* gene, are sensitive to EGFR-targeted drugs gefitinib or erlotinib. However, a lung cancer cell line harboring gene amplification of *c-Met* is sensitive to the c-Met-targeted drug SU11274 but not to EGFR-targeted drugs. C-Met overexpression is identified as one of the bypass mechanisms for acquired resistance to EGFR-targeted drugs. Here we show that the siRNA-mediated knockdown of SNX2 decreases the cell-surface localization of c-Met, but not that of EGFR, resulting in lysosomal degradation of the c-Met protein. SNX2 specifically interacts with c-Met and treatment with lysosomal inhibitors almost completely annihilates downregulation of c-Met protein by SNX2 knockdown. Therefore, silencing of SNX2 markedly alters sensitivity to anticancer drugs targeted to c-Met (SU11274) and EGFR (gefitinib and erlotinib) through promotion of compensatory activation of the EGFR pathway in lung cancer cells. These findings suggest that development of drugs targeting SNX2 could be useful in overcoming drug resistance to EGFR-targeted drugs in lung cancer cells harboring c-Met gene amplification. (*Cancer Sci* 2013; 104: 573–583)

Lung cancer is the most commonly diagnosed malignancy and the most common cause of cancer-related death worldwide.[1] Many tumor types, including non-small-cell lung cancer (NSCLC), demonstrate activated epidermal growth factor receptor (EGFR) expression. The EGFR-targeted drugs, such as gefitinib and erlotinib, have therefore been approved for use in NSCLC treatment. The susceptibility of NSCLC to EGFR-targeted drugs can be regulated by activating somatic mutations of the EGFR kinase domain.[2–4] Moreover, some acquired resistance to EGFR-targeted drugs has been closely associated with overexpression of the receptor tyrosine kinase c-Met through gene amplification,[5] whereas c-Met modifies the sensitivity of cancer cells to gefitinib through human epidermal growth factor receptor (HER) 3-dependent activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway.[6] Overexpression of hepatocyte growth factor (HGF), which is a ligand for c-Met, also markedly alters drug sensitivity to gefitinib in lung cancer cells,[7] whereas c-Met plays an important role in determining the therapeutic efficacy of EGFR-targeted drugs against NSCLC.

The sorting nexin (SNX) family is a diverse group of cytoplasmic and membrane-associated proteins with a common phospholipid-binding motif: the phox homology (PX) domain.[8] The SNX family comprises approximately 33 proteins with a range of biological functions, which play specialized and/or generalized roles in the regulation of protein trafficking, including endosomal trafficking of membrane receptors and transporters.[9] SNX2 shares 63% sequence identity with SNX1 and forms heteromeric complexes with SNX1 and SNX4, whereas SNX1, SNX2, SNX5 and SNX6 together form the mammalian retromer complex.[10,11] Unlike SNX1, SNX2 binds to phosphatidylinositol-3, 4, 5-trisphosphate (PtdIns[3, 4, 5]P_3), with the SNX2 PX domain binding preferentially to PtdIns(3)P.[12] SNX1, SNX2 and SNX6 also interact with receptors for epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin and leptin.[9] SNX1 and SNX2 modulate intracellular membrane trafficking and degradation of EGFR[8,12–14] and SNX6 also promotes its degradation.[15] However, it remains unclear how the membrane trafficking of EGFR and other membrane growth factor receptors is regulated through these SNX.

Gullapalli and colleagues examined the relationship between the cellular expression levels of EGFR or c-Met and SNX and found that activated EGFR co-localizes preferentially with SNX2-positive rather than SNX1-positive endosomes.[14] However, knockdown of SNX1 and SNX2 did not alter EGF/transforming growth factor-α (TGF-α)-induced downregulation of EGFR, suggesting non-essential roles of SNX1 and/or SNX2 in the EGFR degradation pathway.[14] Yeast two-hybrid analysis and co-immunoprecipitation analysis identified SNX2 as an interaction partner of c-Met;[16] however, it is not clear which SNX specifically affects the expression of EGFR and c-Met and how SNX-mediated downregulation or upregulation of growth factor receptors specifically alters the sensitivity of cancer cells to molecular-targeting drugs. Therefore, we investigated whether SNX2 could regulate the expression and localization of c-Met or EGFR family proteins and also could affect the sensitivity of the cells to c-Met- or EGFR-targeted drugs.

Materials and Methods

Cells and reagents. The characteristics of the human lung cancer cell lines (EBC-1, H1993 PC-9 and 11–18) used in the present study have been published previously.[17] EBC-1 and H1993 harbor wild-type EGFR, PC-9 harbors activating mutant EGFR

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(delE746-A750) and 11–18 harbors activating mutant EGFR (L858R). Also, GR5 harboring c-Met amplification was established as a gefitinib-resistant cell line from HCC827 harboring activating mutant EGFR (exon 19 deletion mutation) as described previously.[18] EBC-1 was cultured in DMEM supplemented with 10% FBS and PC-9, 11–18, H1993, HCC827 and GR5 were cultured in RPMI supplemented with 10% FBS. Anti-lysosomal-associated membrane protein (LAMP) 1 was provided by Dr K. Furuta (National Cancer Center Research Institute, Tokyo, Japan). Gefitinib was provided by AstraZeneca Inc. (Macclesfield, UK) and erlotinib was from F. Hoffmann-La Roche Ltd (Basel, Switzerland). Anti-EGFR, pEGFR, pHER3, Akt, pAkt, Erk1/2 and pErk1/2 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-c-Met and anti-HER3 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-HER2, pHER2 and c-Met (for reacting with the amino-terminal domain of c-Met for immunohistochemical analysis) antibodies were from Millipore Corp. (Billerica, MA, USA). Anti-SNX1 and anti-SNX2 antibodies were from BD Bioscience (San Jose, CA, USA). Anti-GAPDH antibody was from Trevigen (Gaithersburg, MD, USA). Anti-transferrin receptor (TfR) antibody was from Invitrogen (Carlsbad, CA, USA). Anti-cathepsin D antibody was from Dako Cytomation (Glostrup, Denmark). The secondary goat anti-rabbit antibody conjugated with Cy3 was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). The Alexa 488-labelled goat anti-mouse secondary antibody was from Molecular Probes (Eugene, OR, USA). SU11274 and MG132 were purchased from Calbiochem (Darmstadt, Germany) and hydroxychloroquine and protease inhibitor cocktail were from Sigma-Aldrich Co. (St Louis, MO, USA). Leupeptin, pepstatin A and E64d were from Peptide Institute, Inc. (Osaka, Japan) and Protein-A coupled Sepharose 4B was purchased from Pharmacia Biotech (Uppsala, Sweden).

Silencing of SNX2 and western blotting. SiRNA corresponding to the nucleotide sequences of SNX1 (5′-AAG AAC AAG ACC AAG AGC CAC-3′) and SNX2#1 (5′-AAG UCC AUC AUC UCC ACC AAG AGC CAC-3′) and negative control siRNA were purchased from Genix Talk (Osaka, Japan). SNX2#2 (HSS110066) and SNX2#3 (HSS186027) were purchased from Invitrogen. C-Met stealth RNAi (HSS106477) was from Invitrogen. SiRNA duplexes were transfected using Lipofectamine RNAiMAX and Opti-MEM medium (Invitrogen) according to the manufacturer's recommendations. At 48 h after siRNA transfection, cells were rinsed with ice-cold PBS and lysed in a lysis buffer (pH 7.5) containing 50 mmol /L Tris-HCl, 0.1% Nonidet P-40 (NP-40), 350 mmol/L NaCl, 50 mmol/L NaF, 1 mmol/L Na_3VO_4, 5 mmol/L EDTA, 1 mmol/L PMSF and 10 μg/mL each of aprotinin and leupeptin. Lysates were subjected to SDS-PAGE and blotted onto Immobilon membranes (Millipore Corp.). After transfer, membranes were incubated with the primary antibody and visualized with the secondary antibody coupled to horseradish peroxidase and ECL western blotting detection reagents (GE Healthcare, Piscataway, NJ, USA). The intensity of luminescence was quantified using a charge-coupled device camera combined with an image analysis system (LAS-4000; Fujifilm, Tokyo, Japan).

Immunoprecipitation. EBC-1 cells were lysed in TBS-T buffer (50 mmol/L Tris-HCl buffer [pH 7.5], 0.15 mol/L NaCl, 1% Triton-X100 and 0.5% deoxycholic acid) containing a protease inhibitor cocktail. After centrifugation for 15 min at 21 500 *g*, the supernatant was used as total cell lysate for either immunoblotting or immunoprecipitation. Protein-A Sepharose 4B was pre-incubated for 2 h at 4°C with appropriate antibodies. The total cell lysate was incubated with the antibody-coupled sepharose for 20 h at 4°C and then washed five times with TBS-T buffer. Immunoprecipitated proteins were washed with lysis buffer several times, eluted with SDS sample buffer and subjected to SDS-PAGE.

Cell viability assay. Exponentially growing cell suspensions (3×10^3 cells/100 μL) were seeded into each well of 96-well microtiter plates. The following day, various concentrations of gefitinib, erlotinib or SU11274 were added. After incubation for 72 h at 37°C, 20 μL of Cell Count Reagent SF (Nacalai tesque, Kyoto, Japan) was added to each well and the plates were incubated for a further 1–2 h at 37°C. Absorbance was measured at 450 nm with a 96-well plate reader. Triplicate wells were tested at each drug concentration. The IC_{50} value (defined as the concentration giving a 50% reduction in absorbance) was calculated from the survival curves.

Real-time quantitative polymerase chain reaction (qPCR). RNA was reverse transcribed using random hexamers and avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA). Real-time qPCR was performed using the Real-time PCR system 7300 (Applied Biosystems, Foster city, CA, USA). In brief, the PCR amplification reaction mixtures (20 μL) contained cDNA, primer pairs, the dual-labelled fluorogenic probe and the Taq Man Universal PCR Master Mix (all from Applied Biosystems). The thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. The relative gene expression for each sample was determined using the formula $2^{\Delta C_t} = 2^{C_t[\text{GAPDH}]-C_t[\text{target}]}$, which reflected the target gene expression normalized to GAPDH levels.

Immunofluorescene microscopy and antibody internalization. EBC-1 cells transfected with siRNA were cultured for 48 h. The cells were treated with the lysosomal protease inhibitors leupeptin (40 μmol/L), pepstatin A (40 μmol/L) and E64d (40 μg/mL) during the final 20 h, fixed in −20°C methanol for 20 min and processed for immunofluorescene analysis with antibodies against c-Met and LAMP1. The secondary goat anti-mouse and anti-rabbit antibodies conjugated with Cyanine Dyes (Cy) 3 or Cy5 were used at 5 mg/mL. Confocal images were obtained through Zeiss 510 meta microscopy (Carl Zeiss Microscopy GmbH, Oberkochen, Germany). For antibody internalization, EBC-1 cells transfected with 20 μmol/L of the indicated siRNA were cultured for 48 h. The cells were then incubated in medium containing anti-c-Met antibody (diluted to 1/10) for 90 min in the presence of lysosomal protease inhibitors, as described above. The cells were then fixed in −20°C methanol for 20 min and processed for immunofluorescene analysis with anti-LAMP1 antibody (green). The internalized antibody to c-Met was detected with a Cy3-conjugated secondary antibody (red).

Results

Expression of growth factor receptors and sensitivity to molecular-targeted drugs in human lung cancer cells. We initially examined the expression levels of growth factor receptors and their phosphorylated forms in the following cultured human lung cancer cell lines: 11–18, harboring the activating mutation L858R; PC-9, harboring the activating EGFR mutation delE746-A750; and EBC-1 and H1993, harboring c-Met gene amplification. The cells showed various expression levels of receptors and their downstream signaling molecules including Akt and Erk 1/2 (Fig. 1A). C-Met was highly expressed in EBC-1 and H1993 cells.

We then compared the sensitivity of these cell lines to gefitinib, which is an inhibitor of EGFR tyrosine kinase, or to SU11274, which is an inhibitor of c-Met tyrosine kinase. Consistent with our previous study,[17] both PC-9 and 11–18 cells were highly sensitive to the cytotoxic effect of gefitinib, with IC_{50} values of 0.03 μmol/L and 1.86 μmol/L, respectively (Fig. 1B), whereas EBC-1 and H1993 were highly sensitive to

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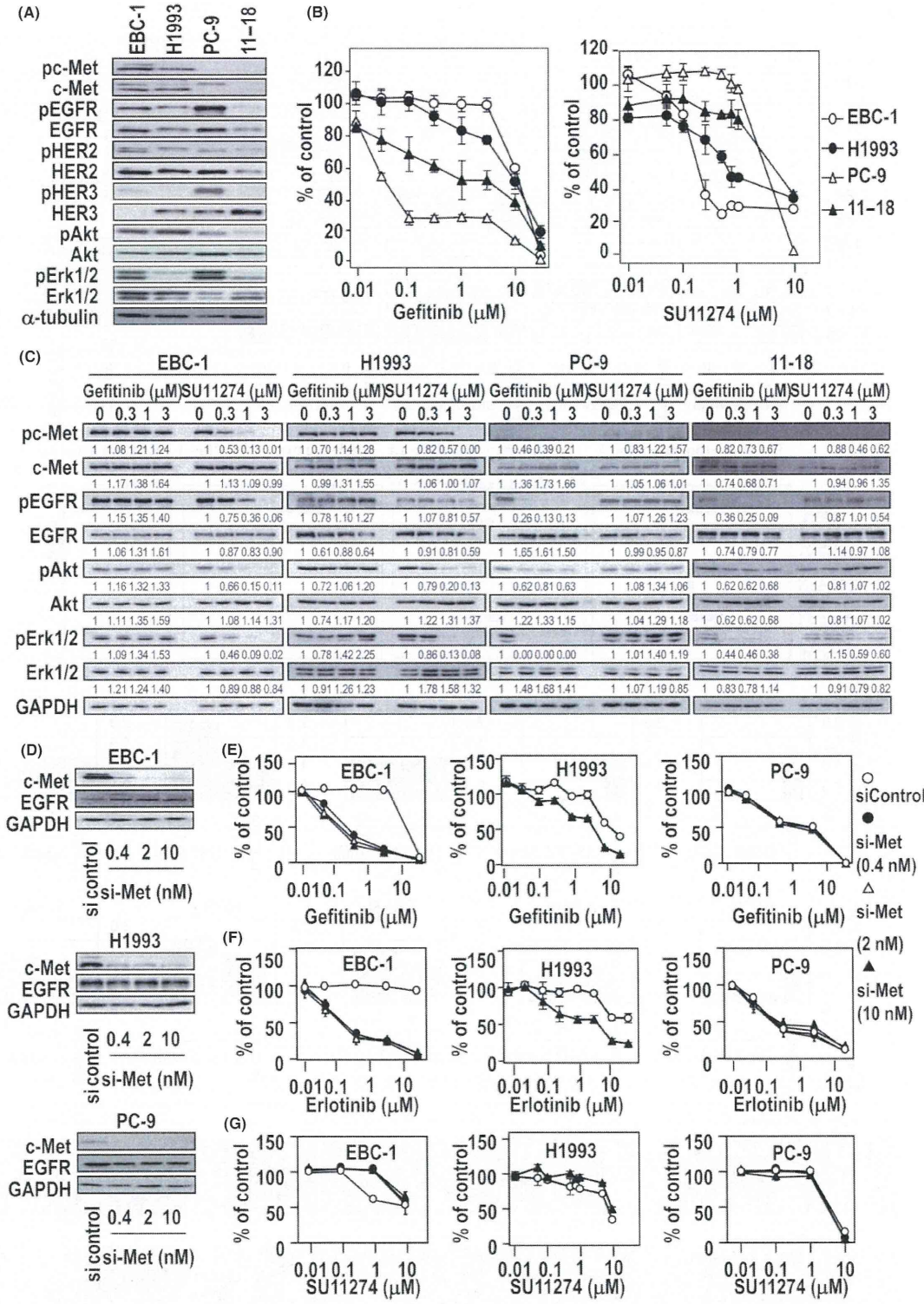

Fig. 1. Expression of growth factor receptors and drug sensitivity to gefitinib (epidermal growth factor receptor [EGFR] tyrosine kinase inhibitor) or SU11274 (c-Met tyrosine kinase inhibitor) in human lung cancer cell lines and altered sensitivities to gefitinib, erlotinib and SU11274 following treatment with c-Met siRNA. (A) Expression of phosphorylated c-Met (pc-Met), c-Met, phosphorylated EGFR (pEGFR), EGFR, phosphorylated human epidermal growth factor receptor (pHER) 2, human epidermal growth factor receptor (HER) 2, pHER3, HER3, phosphorylated protein kinase B (pAkt), protein kinase B (Akt), pErk1/2 and Erk1/2 determined using immunoblotting of protein lysates. Loading control, α-tubulin. (B) Sensitivity of lung cancer cell lines to gefitinib or SU11274 determined using a cell proliferation assay. (C) Effect of gefitinib and SU11274 on expression of growth factor receptors and their downstream signaling molecules determined using immunoblotting. (D) Knockdown of c-Met by c-Met siRNA as determined using immunoblotting. (E–G) Sensitivity of c-Met siRNA-treated lung cancer cell lines to gefitinib (E), erlotinib (F) and SU11274 (G), as determined using a cell proliferation assay.

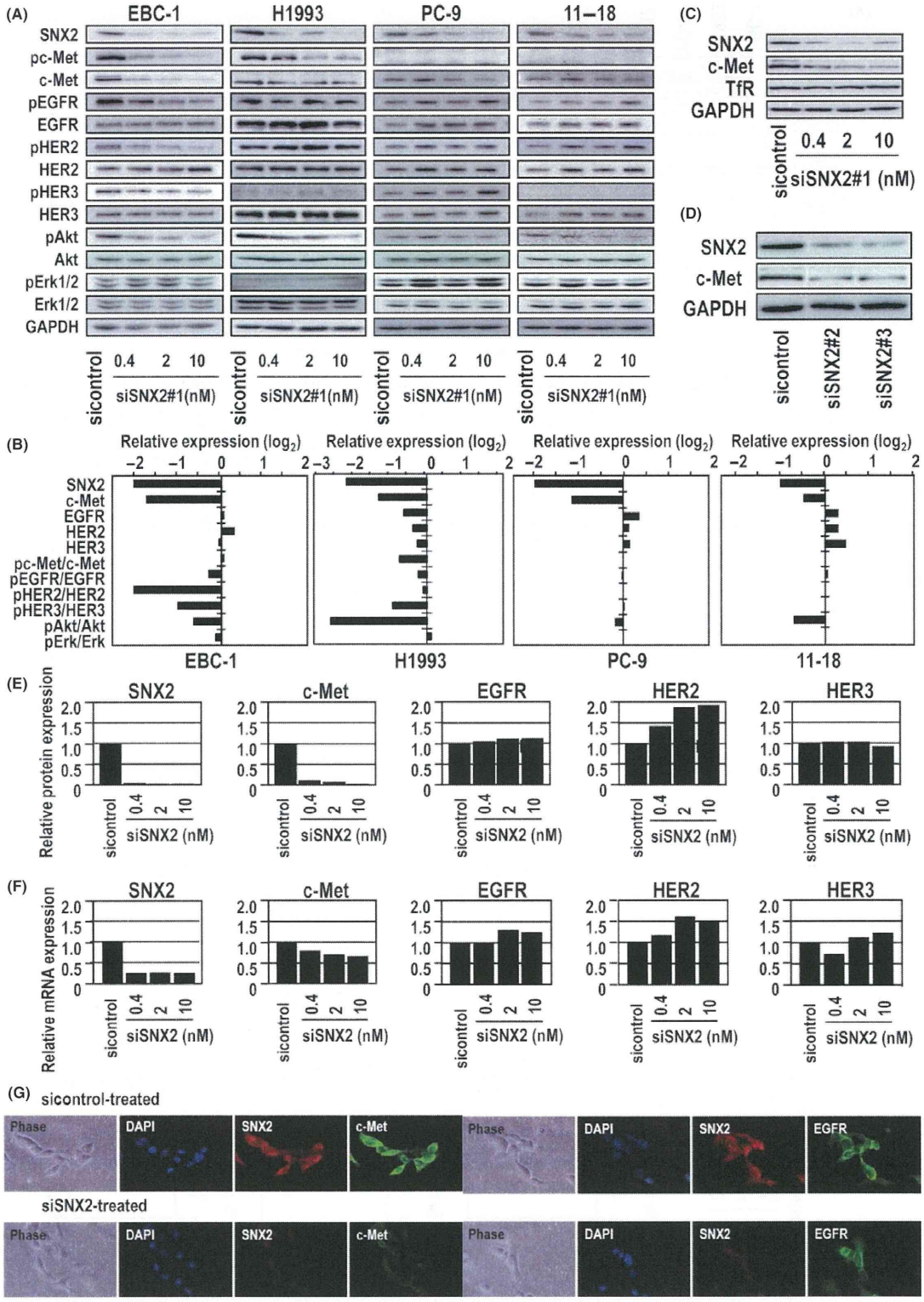

Fig. 2. Effect of sorting nexin (SNX) 2 knockdown on expression of growth factor receptors and downstream signaling molecules. (A) Knockdown of SNX2 by SNX2 siRNA as determined using immunoblotting. (B) Relative protein expression levels normalized to GAPDH. Activities of phosphorylated c-Met, epidermal growth factor receptor (EGFR), human epidermal growth factor receptor (HER) 2, HER3, protein kinase B (Akt) and Erk1/2 normalized to non-phosphorylated forms. (C) Effect of SNX2 knockdown on c-Met and transferrin receptor (TfR) expression in EBC-1 cells as determined using immunoblotting. (D) Effect of SNX2 knockdown using two other SNX2 siRNA on c-Met expression in EBC-1 cells as determined using immunoblotting. (E,F) Comparison of protein and mRNA levels of growth factor receptors in EBC-1 cells treated with SNX2 siRNA. (G) Immunofluorescence analysis of SNX2, c-Met and EGFR expression in EBC-1 treated with SNX2 siRNA. Nuclear-specific staining using DAPI is also shown.

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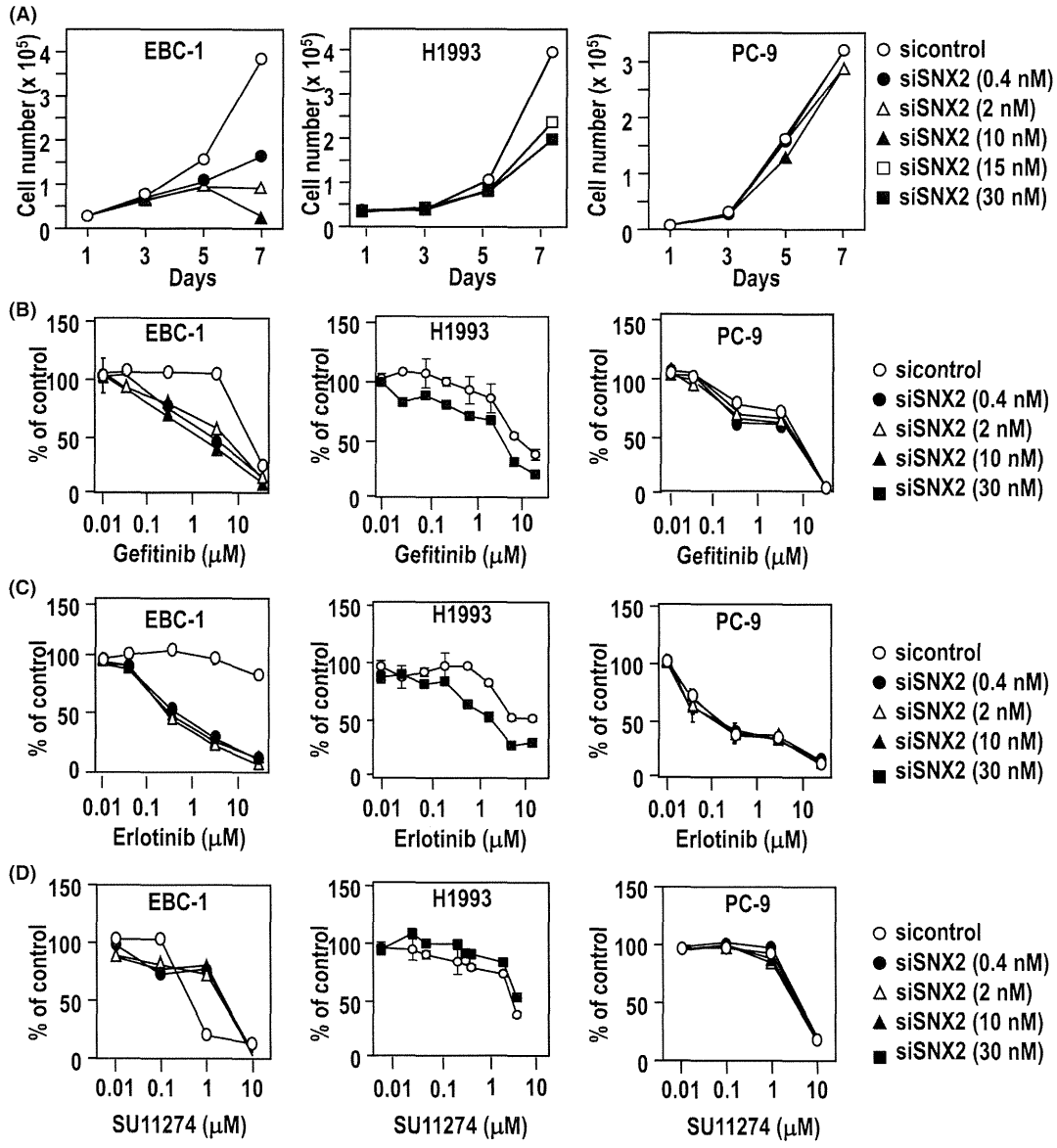

Fig. 3. Altered drug sensitivity to gefitinib, erlotinib and SU11274 after treatment with sorting nexin (SNX) 2 siRNA. (A) Effects of SNX2 knockdown on cell proliferation. (B–D) Sensitivity of SNX2 siRNA-treated lung cancer cell lines to gefitinib (B), erlotinib (C) and SU11274 (D), as determined using a cell viability assay.

SU11274, with IC_{50} values of 0.20 and 0.97 μmol/L, respectively. Gefitinib markedly suppressed the phosphorylation of EGFR, Akt and Erk1/2 in PC-9 and 11–18 but not EBC-1 and H1993 cells (Fig. 1C), consistent with our previous study.[17] In contrast, phosphorylation of c-Met, Akt and Erk1/2 was markedly suppressed by SU11274 in EBC-1 and H1993 cells (Fig. 1C).

We examined whether c-Met knockdown could affect sensitivity to gefitinib, erlotinib or SU11274 in these cell lines. Treatment with c-Met siRNA led to a marked decrease in the expression of c-Met protein in EBC-1, H1993 and PC-9 cells (Fig. 1D). C-Met siRNA sensitized cell proliferation in EBC-1 and H1993 cells to EGFR-targeted drugs (Fig. 1E,F). In contrast, c-Met siRNA decreased sensitivity of EBC-1 and H1993 cells to SU11274 (Fig. 1G), but did not affect the sensitivity of PC-9 cells to these drugs.

Altered expression of growth factor receptor and downstream signaling molecules by SNX2 knockdown in lung cancer cells. Next we examined whether SNX2 knockdown affected the expression of growth factor receptors in the four lung cancer cell lines. Treatment with various doses of SNX2 siRNA (SNX2 siRNA#1) effectively suppressed the expression of SNX2 protein in all four cell lines (Fig. 2A,B). In both EBC-1 and H1993 cells, SNX2 knockdown induced a marked decrease in c-Met expression, but did not decrease expression of EGFR, HER2 or HER3. Also, SNX2 siRNA moderately decreased the expression of c-Met in both PC-9 and 11–18 cells (Fig. 2A,B). However, treatment of EBC-1 cells with SNX2 siRNA did not alter TfR expression (Fig. 2C). Treatment with two other SNX2 siRNA (SNX2#2 and SNX2#3) also suppressed expression of both SNX2 protein and c-Met protein in EBC-1 cells (Fig. 2D). Therefore, we used SNX2

siRNA#1 in all experiments and herein describe it as SNX2 siRNA.

In EBC-1 cells, SNX2 knockdown reduced the expression of SNX2 protein and mRNA by at least 80% compared with the control (Fig. 2E,F). Surprisingly, SNX2 siRNA treatment blocked the expression of c-Met protein almost completely (Fig. 2E), whereas the expression of c-Met mRNA was blocked by only 20–30% (Fig. 2F). However, there was no apparent effect on either the mRNA or protein expression of the EGFR family. These results suggest that the suppression of c-Met expression by SNX2 knockdown is mainly attributable to the loss of c-Met protein rather than mRNA expression. Immunofluorescene analysis confirmed that SNX2 siRNA almost completely inhibited c-Met but not EGFR expression (Fig. 2G).

Effect of SNX2 knockdown on cell proliferation and sensitivity to molecular-targeted drugs in lung cancer cells. Next we examined whether SNX2 knockdown affected cell proliferation in EBC-1, H1993 and PC-9 cells. SNX2 siRNA treatment strongly inhibited the proliferation of EBC-1 cells, but had little or no effect on PC-9 cells (Fig. 3A). Sensitivity to gefitinib was increased by SNX2 siRNA in EBC-1 and H1993 cells, but not in PC-9 cells (Fig. 3B). Similarly, the sensitivity of EBC-1 and H1993 cells to erlotinib was increased by SNX2 knockdown (Fig. 3C). In contrast, SNX2 knockdown decreased the sensitivity of EBC-1 and H1993 cells to SU11274, but did not affect the sensitivity of PC-9 cells (Fig. 3D). However, the effect of SNX2 knockdown on cellular sensitivity to molecular-targeted drugs was more marked in EBC-1 cells than H1993 cells and that in 11–18 cells was almost similar to PC-9 cells (data not shown). Taken together, these results suggest that the proliferation of EBC-1 and H1993 cells is more dependent on the c-Met pathway than that of PC-9 cells.

We then compared protein expression with or without gefitinib under SNX2 knockdown conditions. Compared with the control siRNA, phosphorylation of Akt and Erk1/2 was markedly suppressed by gefitinib when treated with SNX2 siRNA (Fig. 4). Activation of downstream regulatory molecules thus seems to be highly susceptible to EGFR-targeted drugs when the c-Met-driven signaling pathway is blocked by SNX2 knockdown.

The structure and protein-trafficking function of SNX2 are similar to those of SNX1. Therefore, we investigated whether SNX1 affects c-Met expression and sensitivity to EGFR-targeted or c-Met-targeted drugs in the same way as SNX2. Treatment with SNX1 siRNA effectively decreased the expression of SNX1 but not SNX2 in EBC-1 cells (Fig. S1A). Knockdown of SNX1 did not affect the expression of c-Met or EGFR in either EBC-1 or PC-9 cells (Fig. S1B) or the sensitivity to EGFR-targeted drugs or SU11274 (Fig. S1C–E).

Inhibition of lysosomal function restores c-Met downregulation by SNX2 knockdown. Next we examined whether the SNX2 protein directly interacts with the c-Met protein. In EBC-1 cells, co-immunoprecipitation of c-Met and SNX2 proteins was revealed (Fig. 5A), consistent with the findings of a previous study by Schaaf *et al.*[16] In contrast, no co-immunoprecipitation of c-Met proteins and SNX1 was observed following immunoblotting with a SNX1-specific antibody (Fig. 5A).

We examined whether various inhibitors of membrane trafficking could restore the downregulation of c-Met by SNX2 siRNA. Treatment with the calcium ionophore A23187 and the matrix metalloproteinase inhibitor GM6001 did not restore SNX2 siRNA-induced downregulation of c-Met protein (data not shown). The proteasome inhibitor MG132 partially restored the expression of SNX2 protein without affecting that of c-Met protein (data not shown). As shown in Figure 5(B,C), treatment with the endosome/lysosome inhibitor leupeptin, pepstatin A, E64d or hydroxychloroquine markedly restored c-Met but not SNX2 expression; cathepsin D expression was also

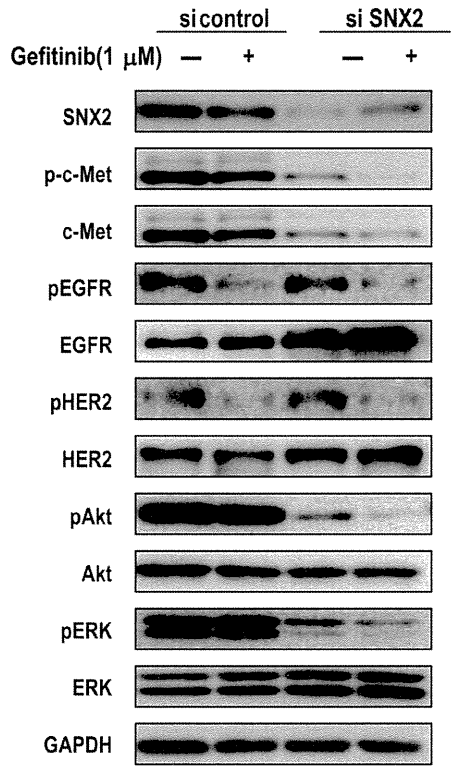

Fig. 4. Effect of gefitinib on activation of growth factor receptors and the downstream signaling molecules in sorting nexin (SNX) 2-silenced EBC-1 cells. Effects of gefitinib on expression of epidermal growth factor receptor (EGFR), human epidermal growth factor receptor (HER) 2, protein kinase B (Akt) and Erk1/2 and the phosphorylated molecules in SNX2-silenced cells determined using immunoblotting analysis. HER, human epidermal growth factor receptor; pAkt, phosphorylated protein kinase B; pc-Met, phosphorylated c-Met; pEGFR, phosphorylated EGFR; pHER2, phosphorylated human epidermal growth factor receptor 2.

restored using this treatment because maturation of cathepsin D is known to be processed in lysosome.

We further examined whether an endosome/lysosome inhibitor could annihilate the inhibitory effect of an EGFR-targeted drug on cell proliferation. Treatment with hydroxychloroquine was found to reduce the inhibitory effect of erlotinib under SNX2 knockdown conditions in EBC-1 cells compared with control conditions (Fig. 5D). Under SNX2 knockdown conditions, the inhibition of lysosome function thus restored c-Met expression, resulting in cancellation of the sensitization to erlotinib.

Localization of c-Met in the lysosomal compartment by SNX2 knockdown in the presence of lysosome inhibitors. We further examined which compartment was responsible for the degradation of c-Met under SNX2-knockdown conditions. As c-Met expression was almost completely inhibited following SNX2 knockdown, we used the lysosomal protease inhibitors leupeptin, pepstatin A and E64d to examine the localization of c-Met using confocal microscopy (Fig. 6). Under control conditions (control siRNA), c-Met exclusively localized to the cell surface, but could not readily be detected in the intracellular compartments such as late endosomes and lysosomes, as indicated by LAMP1 even in the presence of lysosomal protease inhibitors (Fig. 6A). In contrast, following SNX2 siRNA transfection, c-Met was not expressed on cell-surface membranes but was localized to the late endosomes and lysosomes in the presence of lysosome

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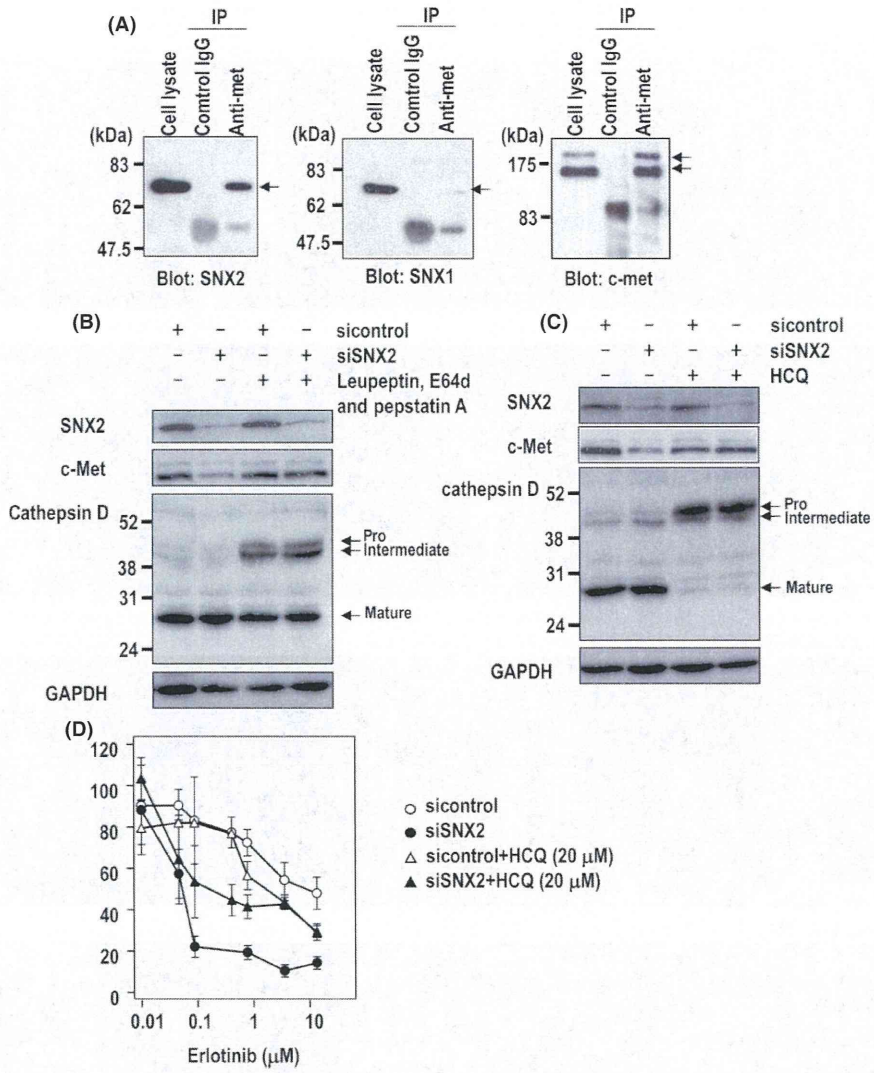

Fig. 5. Co-immunoprecipitation (IP) of sorting nexin (SNX) 2 and c-Met and the effect of lysosomal inhibitors on expression of c-Met by SNX2 knockdown in EBC-1 cells. (A) Immunoprecipitation with anti-c-Met antibody and blotting with anti-SNX1, SNX2 and c-Met antibody. (B,C) Effect of lysosome inhibitors on c-Met expression with or without SNX2 siRNA. (B) EBC-1 cells were transfected with siRNA, cultured for 48 h and treated with lysosome inhibitors during the final 20 h. (C) Cells were transfected with siRNA, cultured for 48 h and treated with hydroxychloroquine (HCQ) during the final 42 h. Protein extracts were resolved using immunoblotting and probed with various antibodies. (D) Cells were transfected with siRNA, cultured for 24 h and treated with erlotinib and HCQ. After 72 h, the number of viable cells was counted.

inhibitors (Fig. 6A, arrows). Incubation with the anti-c-Met antibody following control siRNA resulted in exclusive retention of the antibody at the cell surface, suggesting that there was little internalization and trafficking of c-Met to late endosomes and lysosomes (Fig. 6B). However, under SNX2-silencing conditions, the internalized antibody co-localized with LAMP1 (Fig. 6B, arrows). Together, these results suggest that the c-Met protein is internalized from the cell surface and transported to late endosomes and lysosomes when SNX2 is depleted.

Overcoming the effect of gefitinib resistance in a lung cancer cell line harboring both activated EGFR mutation and c-Met amplification by SNX2 knockdown. Last, we examined whether SNX2 knockdown overcomes drug resistance in lung cancer cell line GR5 cells harboring c-Met amplification and activation mutation of EGFR.[18] As shown in Figure 7(A), GR5 cells showed higher drug resistance to gefitinib and also to SU11274, while parental HCC827cells showed higher sensitivity to gefitinib and higher resistance to SU11274. Also, GR5 cells showed enhanced expression of c-Met and HER3 compared with HCC827 cells (Fig. 7B). Treatment with SNX2 siRNA decreased expression of c-Met but not expression of EGFR and HER3 (Fig. 7C). However, gefitinib markedly suppressed Akt phosphorylation under SNX2 knockdown conditions in GR5 cells. Cellular sensitivity to gefitinib was increased by SNX2 siRNA and also by c-Met siRNA in GR5 cells (Fig. 7D). Thus, SNX2 knockdown seems to overcome drug resistance to gefitinib in cancer cells harboring c-Met amplification.

Discussion

The present study demonstrated that expression of c-Met was specifically downregulated by SNX2 knockdown in four human lung cancer cell lines. This was accompanied by suppression of the activation of PI3K/Akt in EBC-1 and H1993 but not PC-9 or 11–18 cells (Fig. 2A). Of these four lung cancer cell lines, c-Met was amplified with wild-type