MEX3A promotes the proliferation, migration, and invasion of non-small-cell lung cancer by activating the MEK/ERK pathway.

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MEX3A promotes the proliferation, migration, and invasion of non-small-cell lung cancer by activating the MEK/ERK pathway

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12 **Keywords:** MEX3A, non-small-cell lung cancer, MEK/ERK pathway, potential therapeutic
13 target gene, biological behavior

14 **Abstract**

15 **Background:** Lung cancer is the second most prevalent type of cancer worldwide; however, its death
16 is the highest among all cancers. Previous studies have shown that MEX3A is associated with
17 Argonaute (Ago) and CDX2 proteins, which are important in tumor progression. High expression of

18 MEX3A is also known to negatively affect the therapeutic impact of radiotherapy and chemotherapy.
19 Therefore, we investigated the role of MEX3A in non-small cell lung cancer (NSCLC).

20 **Methods:** Tumor specimens were collected from 87 NSCLC patients who underwent surgical
21 resection between 2014 and 2019 at the Pathology Archive of the First Affiliated Hospital of China
22 Medical University, and were subsequently used for immunohistochemistry. NSCLC cell lines and
23 normal bronchial epithelial HBE cell line were used to study the effects of MEX3A by utilizing
24 MEX3A-siRNA, MEX3A-plasmids, and the ERK pathway inhibitor U0126. Western blotting,
25 colony formation assays, MTT assays, and Transwell assays with or without Matrigel were
26 performed to explore the impact of MEX3A on NSCLC.

27 **Results:** MEX3A protein expression was associated with tumor differentiation ($P=0.044$), size
28 ($P=0.020$), lymph node status ($P=0.001$), and p-TNM stage ($P=0.009$) and was preferentially
29 expressed in tumor tissues. MEX3A was found to be localized to the nucleus. The expression level
30 in cancer tissue was significantly higher than that in normal tissue. According to our study, MEX3A
31 was shown to promote the proliferation, migration, and invasion of NSCLC. In addition, MEX3A
32 regulated the expression of P-MEK, P-ERK, CyclinA2, CDK4, CDK6, RhoA and MMP2. The MEK
33 inhibitor U0126 reduced the biological effects of MEX3A, suggesting that MEX3A increases the
34 carcinogenic activity of NSCLC cells through activation of the MEK/ERK signaling pathway.

35 **Conclusion:** These results suggest an association between MEX3A expression and the unfavorable
36 clinicopathological characteristics of NSCLC patients. And MEX3A promote NSCLC progression by
37 increasing the proliferative and metastatic potential of cancer cells through upregulation of the
38 MEK/ERK pathway. We propose that MEX3A is a candidate prognostic biomarker and potential
39 therapeutic target in NSCLC.

40 **Keywords:** MEX3A, non-small-cell lung cancer, MEK/ERK pathway, therapeutic target gene

1 Background

Lung cancer is the second most prevalent type of cancer worldwide; however, the death rate of lung cancer is the highest among all cancers. Non-small-cell lung carcinoma (NSCLC) accounts for 85% - 90% of lung cancer cases (1-3). If surgical resection can be performed in a timely manner, it is the best treatment for NSCLC patients. However, even when patients are treated in time, their long-term survival rate is not ideal, and many patients will still die from recurrence or metastasis. The prognosis of NSCLC patients is mainly affected by metastasis and is closely related to the transcriptional regulation of key genes (4-6).

The MEX3-family of proteins are translation inhibitors, that are necessary for the recognition and maintenance of germ line stem cells (7). The MEX-3-family has four members encoded by the following genes: *MEX3A*, *MEX3B*, *MEX3C*, and *MEX3D*, which are located on chromosomes 1q22, 15q25.2, 18q21.1 and 19p13.3, respectively (7). These proteins have two domains: one is a highly conserved RNA-binding domain (KH domain), the other is ring domain with E3 ubiquitin ligase activity located at the C-terminal (8). These four proteins are concentrated primarily in the cytoplasm, and they shuttle between the cytoplasm and nucleus through the CRM1 pathway (9). MEX3D is widely expressed in a variety of tissues, while the other three proteins are differentially expressed depending on tissue-type. MEX3A and MEX3D are components of the RNA granules known as processing bodies (P-bodies) and locate and interact with hDcp1, a decapping factor (7). During the degradation of mRNA, decapitation at the 5' end is primarily carried out at a specific site in the cytoplasm known as a P-body. MEX3A and MEX3B are related to Argonaute (Ago) (10), of which two domains, PAZ and PIWI, are essential for the interaction between siRNA and target mRNA, leading to the cutting or translation inhibition of target mRNA. MEX3C, an RNA-binding E3 ubiquitin ligase, mediates the decay of post-transfer HLA-A antibodies.(11). In regard to tumors,

64 MEX3A is related to the occurrence and development of gastric and colorectal cancer. Moreover,
65 MEX3A has been shown to affect the efficacy of radiotherapy and chemotherapy (12-14).

66 Until now, the relationship between MEX3A and the development of NSCLC has not been studied.
67 Our study is the first to demonstrate the significance of MEX3A in the poor prognosis of NSCLC.

68 **2 Materials and Methods**

69 **2.1 Patients and specimens**

70 A total of 87 patients with NSCLC documented between 2014 and 2019 in the Pathology Archive of
71 the First Affiliated Hospital of China Medical University were enrolled in our study. These patients
72 were first diagnosed between 2014 and 2019 and underwent surgical resection of tumors. None of the
73 patients underwent radiotherapy or chemotherapy prior to surgical resection. Tumor tissues and
74 corresponding normal adjacent tissues were stored in -80°C prior to analysis.

75 **2.2 Cell culture**

76 Along with the tissues obtained from NSCLC patients, we used NSCLC cell lines A549, H1299,
77 H460, H661, SK-MES-1, H226, and H292 (Cell Bank of the China Academy of Sciences, Shanghai,
78 China), and normal bronchial epithelial HBE cell lines A549, 1299, H460, H661, H226 (ATCC :
79 Manassas, VA, USA) cultured with RPIM 1640 medium (Gibco, Waltham, MA, USA). HBE cells
80 were cultured with MEM (Gibco) containing 1.5g/L NaHCO₃ and 0.11 g/L sodium pyruvate. HBE
81 and H292 cells were cultured with DMEM (Gibco) containing 1.5g/L NaHCO₃. All media were
82 supplemented with 10% qualified fetal bovine serum (FBS15015: Clark Biosciences Richmond, VA,
83 USA).

84 **2.3 Cell transfection and inhibitor**

85 Following a 20 h incubation period, MEX3A-siRNA (Ribobio, Guangzhou, China) and MEX3A-
86 plasmids (GeneCopoeia, USA) were transfected into the cell suspension together with opti-MEM
87 (Gibco, Waltham, MA, USA) and Lipofectamine 3000 (Invitrogen, Waltham, MA, USA) to decrease
88 or increase the expression of MEX3A in NSCLC cells.

89 Cells were treated with 10 μ M U0126 (MedChemExpress, Monmouth Junction, NJ, USA), an
90 inhibitor that blocks the mitogen-activated protein kinase kinase/extracellular receptor kinase
91 (MEK/ERK) signaling pathway. U0126 was dissolved in dimethyl sulfoxide (DMSO) (10 μ M) and
92 added for 1 h after 48 h transfection, with the same volume of DMSO added to control cells.

93 **2.4 Immunohistochemistry**

94 The specimens were fixed in 10% neutral formalin and embedded in paraffin. Then, the specimens
95 were cut into 4 μ m thick sections. Immunohistochemistry was performed according to the
96 streptavidin-peroxidase method. Firstly, the sections were deparaffinized in xylene, and rehydrated in
97 a graded alcohol series for dewaxing. The sections were washed by phosphate-buffered saline (PBS).
98 Then, sections were treated with 0.01 mol/L citrate buffer (Maixin-Bio, Fuzhou, China) under high
99 pressure for 3 min. An Elivision Reagent KIT9921 (Maixin, Fuzhou, China) for
100 immunohistochemistry was used according to the manufacturer's instructions. Sections were treated
101 with antibody-MEX3A (1:200 dilution, Sigma, St Louis, MO, USA) for 16 h. The reaction was
102 visualized using the Elivision super HRP (Mouse/Rabbit) IHC Kit (Maixin, Fuzhou, China). The
103 chromogenic reagent was 3'3'-diaminobenzidine and the nuclei were identified with hematoxylin
104 (Maixin, Fuzhou, China).

105 Two investigators, blinded to the clinical data, semi-quantitatively scored all slides by evaluating the
106 staining intensity and percentage of cells stained in representative areas of each slide. The
107 percentages of stained cells were scored as follows: 1 (1%-25%), 2 (26%-50%), 3 (51%-75%), 4

108 (76%-100%). Based on staining intensity, MEX3A expression was also scored as follows: 0 (no
109 staining), 1 (weak staining), 2 (moderate staining), and 3 (high staining). Percentage scores were
110 assigned as follows 0 (0%), 1 (1%-30%), 2 (31%-70%), and 3 (71%-100%). The scores of each
111 tumor sample were multiplied to give a final score ranging from 0 to 9, with tumor samples scoring >
112 3 considered MEX3A-high, and those scoring ≤ 3 considered MEX3A-low.

113 **2.5 Western Blotting**

114 The expression of proteins directly influences cell migration and invasion (15-19) as well as cell
115 proliferation and cell cycle progression (20-24). Total cellular or tissue protein was extracted using a
116 lysis buffer (P0013, Beyotime, Biosciences, Shanghai, China) and added into a protease-inhibitor
117 cocktail (B14002, Biotool, Shanghai, China) and phosphatase-inhibitor cocktail (B15002, Biotool).
118 Proteins (80 μ g/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis
119 (10% gels), and were then transferred to polyvinylidene fluoride membranes (Millipore, Billerica,
120 MA, USA), and the membranes were blocked with 5% skim milk (232100; Becton Dickinson,
121 Franklin Lakes, NJ, USA) in Tris-buffered saline with Tween-20 at room temperature for 1 h. After
122 being treated with the appropriate primary antibodies (Table 1) at 4°C for 16 h, the proteins were
123 treated with horseradish peroxidase-conjugated anti-mouse/rabbit IgG (1:200, ZSGB-BIO, Beijing,
124 China) at 37°C for 1 h. Immune reactivity was detected by enhanced chemiluminescence (Thermo
125 Fisher Scientific, Waltham, MA, USA) using a BioImaging system (UVP, Inc., Upland, CA, USA).
126 Relative protein expression was calculated after normalization to GAPDH or β -actin used as a
127 loading control. The data were visualized and analyzed in ImageJ software.

128 **2.6 Cell proliferation and colony formation assays**

129 Cell proliferation was evaluated daily for 5 days using the colorimetric MTT assay. For this assay,
130 96-well culture plates were seeded with 3000 cells/100 μ L culture medium with 10% qualified fetal

131 bovine serum in each well. MTT solution (10 μ L/well) was added for 4 h, and then MTT crystals in
132 living cells were solubilized with 100 μ L DMSO and the optical density was measured at 490 nm
133 using a microplate reader.

134 For colony formation assays, cells were seeded in 6-well culture plates at a density of 500 cells/dish
135 and incubated for 8-12 days. The cells were washed with PBS, stained with Giemsa solution, and
136 colored with hematoxylin. Colonies with more than 50 cells were counted. At least 3 independent
137 experiments were carried out under identical conditions.

138 **2.7 Cell migration and invasion analysis**

139 Cell migration and invasion assays were carried out in 24-well Transwell chambers containing inserts
140 with a pore size of 8 μ m (Costar, Washington, DC, USA). For invasion assays, the inserts were
141 treated with 100 μ L Matrigel (1:9 dilution; BD Biosciences). 5×10^5 cells were seeded in the inserts,
142 and the cells on the upper membrane surface of the inserts were removed to the bottom membrane
143 surface after 24 h. Cells were fixed with polyformaldehyde and stained with hematoxylin, and then
144 washed with PBS.

145 **2.8 Statistical analysis**

146 SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. A Kaplan-Meier
147 estimator was used to analyze the prognosis of patients (25-26). Correlation between MEX3A
148 expression and clinicopathological features were examined by chi-squared tests, and the differences
149 between cell groups were compared by paired t-tests. Two-tailed P-values < 0.05 were considered
150 statistically significant.

151 **3 Results**

152 **3.1 Patient demographics and clinicopathological characteristics**

Of the 87 patients enrolled in our study, 63 were men and 24 were women, all between the ages of 37 and 77. According to the seventh edition of the International Union against Cancer (UICC) Tumor, Node, Metastasis (TNM) Staging System for Lung Cancer, patients were categorized as having stage I (n=29), II (n=19), or III (n=39) lung cancer. The histological diagnoses and differentiation grades of the tissue samples were evaluated according to the World Health Organization (WHO) classification system as either adenocarcinoma (n=40) or squamous cell carcinoma (n=47). Cancer cells were highly differentiated in 31 patients and moderately or poorly differentiated in the remaining 56. Lymph node metastases were present in 45 patients. In 40 patients, the tumor was smaller than 3 cm.

3.2 MEX3A is highly expressed in NSCLC and correlated with poor prognosis

Analysis of MEX3A protein expression by immunocytochemistry of tumor samples from 87 patients with NSCLC indicated that the expression of MEX3A in tumor tissues was significantly higher than in the interstitium, bronchus, and alveoli. We also found that MEX3A was located in nuclei (Figure 1A). Western blots detected the expression of MEX3A in tumor tissues and paracancerous tissues, the results were consistent with those of immunocytochemistry (Figure 1B). Analysis of MEX3A protein expression levels as they relate to clinical parameters, we found that MEX3A levels correlated with differentiation ($P=0.044$), tumor size ($P=0.020$), lymph node metastasis ($P=0.001$), and p-TNM stage ($P=0.009$), but did not vary significantly with age ($P=0.295$), gender ($P=0.473$), or tumor histological type ($P=0.838$) (Table 2). Kaplan-Meier analysis demonstrated that MEX3A is statistically significant in the poor prognosis of NSCLC (Figure 1D).

Furthermore, western blotting of MEX3A protein expression in NSCLC cell lines and normal bronchial epithelial HBE cells was conducted (Figure 1C). H661, and H292 showed the highest MEX3A expression levels; H460 showed the lowest expression level; and A549 and H1299 were in

the middle. Therefore, A549 and H1299 cells were used for subsequent experiments to explore the impact of MEX3A in NSCLC.

3.3 MEX3A promotes the proliferation of NSCLC.

In order to investigate whether MEX3A regulates the proliferation of NSCLC, we transfected A549 and H1299 cells with either MEX3A-siRNA to knockdown MEX3A expression or MEX3A-plasmids to increase its expression. When MEX3A expression decreased by 50% or increased by 100%, transfection was considered to have been effective. The colony forming ability of A549 cancer cells was inhibited in cells treated with MEX3A-siRNA ($P=0.0002$) and enhanced in those transfected with MEX3A-plasmids when compared to control ($P=0.0007$). Similar results were obtained in the H1299 cell line ($P=0.0004$ and $P=0.0008$, respectively) (Figure 2A). Next, the results of an MTT assay demonstrated that the proliferation ability of cells treated with MEX3A-siRNA was reduced, whereas this ability was strengthened in cells treated with MEX3A-plasmids in both A549 and H1299 cells when compared with control (A549: $P=0.0056$ and $P=0.0011$; H1299: $P=0.0002$ and $P=0.0003$) (Figure 2B).

Finally, in western blots of A549 cells treated with MEX3A-siRNA or MEX3A-plasmids, we found that compared with control cells, the expression of Cyclin2 ($P=0.0082$ and $P=0.048$, respectively), CDK4 ($P=0.0151$ and $P=0.0092$, respectively), and CDK6 ($P=0.0024$ and $P=0.0001$, respectively) was decreased in MEX3A-siRNA treated cells, and increased in cells transfected with MEX3A-plasmids (Figure 2C). Similar results were obtained in H1299 cells (Cyclin2: $P=0.0052$ and $P=0.0002$; CDK4: $P=0.0462$ and $P=0.0002$; CDK6: $P=0.0157$ and $P=0.0027$; all values reflect MEX3A-siRNA and -plasmid, respectively) (Figure 2D). These results indicate that MEX3A can promote the proliferation of NSCLC.

3.4 MEX3A promotes the migration and invasion of NSCLC

In order to investigate whether MEX3A can regulate the migration and invasion of NSCLC, we once again transfected A548 and H1299 cells with either MEX3A-siRNA or MEX3A-plasmids. In Transwell assays without Matrigel, cells treated with MEX3A-siRNA demonstrated a weaker migration ability (A549: $P=0.0047$; H1299: $P=0.0032$), and cells transfected with MEX3A-plasmids demonstrated a stronger migration ability (A549: $P=0.0006$; H1299: $P=0.0003$). Similar results were obtained for both cell lines in Transwell assays with Matrigel, suggesting that MEX3A promotes the invasion ability of cancer cells (A549: $P=0.00403$ and $P=0.0002$; H1299: $P=0.0029$ and $P=0.0005$; values reflect MEX3A-siRNA and -plasmid, respectively) (Figure 3A).

Finally, western blots of A549 cells transfected with MEX3A-siRNA and MEX3A-plasmids demonstrated decreased expression of RhoA and matrix metalloproteinase 2 (MMP2) in MEX3A-siRNA treated cells (RhoA: $P=0.0146$; MMP2: $P=0.0147$), and increased expression in cells transfected with MEX3A-plasmids when compared with control (RhoA: $P=0.0453$; MMP2: $P=0.0273$) (Figure 3B). Similar results were obtained for H1299 cells (Figure 3C) (RhoA: $P=0.0003$ and $P=0.0007$; MMP2: $P=0.0012$ and $P=0.0004$). These results indicate that MEX3A promotes the migration and invasion of NSCLC.

3.5 MEX3A activates the MEK/ERK pathway

In order to explore how MEX3A regulate NCSLS, we used western blots of A549 cells to determine the expression levels of proteins in signal transduction pathways. Compared with control cells, the expression of phosphorylated-ERK (P-ERK) and phosphorylated-MEK (P-MEK) decreased in MEX3A-siRNA treated cells (P-ERK: $P=0.0009$; P-MEK: $P=0.0028$), whereas expression of P-ERK and P-MEK increased in cells transfected with MEX3A-plasmids (P-ERK: $P=0.0005$; P-MEK: $P=0.0002$). The expression of ERK was unaffected in both treatments when compared to control (Figure 4A). Similar results were achieved in H1299 cells (Figure 4B) (P-ERK: $P=0.0226$ and

221 P=0.0002; P-MEK: P=0.0053 and P=0.0007; values reflect MEX3A-siRNA and -plasmid,
222 respectively).

223 **3.6 MEX3A promotes the biological behavior of NSCLC by activating the MEK/ERK** 224 **pathway**

225 To investigate whether MEX3A can promote the biological behavior of NSCLC through the
226 MEK/ERK signaling pathway, MEX3A-plasmid transfected A549 and H1299 cells were treated with
227 DMSO or U0126 (an ERK pathway inhibitor). The colony forming ability of MEX3A-plasmid
228 transfected cells was inhibited when treated with U0126 (A549: P=0.0007 and P =0.0009; H1299:
229 P=0.0002 and P=0.0002; for each group values are control vs. transfection and transfection vs.
230 transfection + U0126, respectively) (Figure 5A). Similar results were observed in MTT assays and
231 Transwell assays both with and without Matrigel (Table 3) (Figure 5B and C). These results suggest
232 that U0126 inhibited the proliferation, migration, and invasion of cancer cells, which further suggests
233 that MEX3A promotes the proliferation, migration, and invasion of NCSLS by activating the
234 MEK/ERK pathway.

235 **4 Discussion**

236 In the present study, we have demonstrated that MEX3A can promote the progression of NSCLC.
237 MEX3A was mainly localized to the nucleus of all tissues, and its expression was enhanced in
238 NSCLC tissues. Moreover, MEX3A expression was positively correlated with the differentiation of
239 NSCLC, the size of cancer nests, whether there was lymph node metastasis or not, and pTNM. In our
240 study, we demonstrated that MEX3A can promote the proliferation, migration, and invasion of
241 NSCLC cells. To explore the mechanism by which MEX3A promotes the biological behavior of
242 NSCLC, we determined the expression of cyclin related proteins, cyclin-binding proteins, and
243 migration and invasion related proteins. Finally, we screened for protein expression associated with

244 several tumor related pathways such as AKT, NF- κ B, NOTCH, and mTOR, and found that the
245 expression of proteins in the MAPK pathway were influenced by MEX3A levels. However, the
246 relationship between MEX3A and MAPK had not been previously explored.

247 MAPK is an evolutionarily conserved signal regulator, which regulates many cellular functions.
248 There are four members of the MAPK family, ERK1/2, JNK, p38, and ERK5. ERK1/2 is the primary
249 contributor to the MAPK signaling pathway, which is the most important signal transduction
250 pathway between cell membrane receptors and the intracellular environment. MAPK signaling plays
251 an important role in the development of a variety of malignant tumors (29). Our research focused on
252 the regulatory effects of MEX3A on ERK1/2. ERK1/2 is phosphorylated following the activation of
253 RAS, RAF, and MEK (30,31). Once phosphorylated, ERK1/2 is activated and transmits extracellular
254 signals, causing a series of effects, including cell proliferation, apoptosis, differentiation, invasion,
255 and metastasis, thus promoting the occurrence and development of tumors (32).

256 It has been shown that in gastric cancer cells, knockdown of MEX3A with siRNA can inhibit the
257 proliferation and migration of cancer cells, but the mechanism by which this occurs unclear (14).

258 MEX3A has also been shown to have a negative regulatory effect on CDX2, a homeobox
259 transcription factor, in Caco-2 and AGS cells (13). MEX3A inhibits the expression of CDX2 in the
260 gastrointestinal environment, which affects intestinal differentiation, polarity, and stem cell
261 characteristics, which are key mechanisms of tissue homeostasis (13). MEX3A interacts with the
262 MEX-3 recognition element (MRE) located in the 3'-untranslated region of CDX2 mRNA (13). We
263 can predict whether MEX3A can interact with the mRNA of proteins in the MAPK pathway by
264 screening for the sequence of this MEX-3 recognition element. Previous studies have shown that
265 MEX3A is a component of P bodies and interacts with hDcp1a, a decapping factor (27). P bodies are
266 the sites of RNA decay and storage of untranslated transcript (27). Therefore, MEX3A may be
267 regulate the mRNA of MAPK pathway genes by forming P bodies, thus affecting the development of

NSCLC. Moreover, MEX3A interacts with Ago proteins (28); Ago1 and Ago2 are the key components of silencing RNA in, which are closely related to tumor development. Therefore, through its interaction with Ago proteins, MEX3A may affect the transcription of MAPK pathway related proteins. Previous studies on colorectal cancer have shown that high expression of MEX3A confers resistance to chemotherapy and γ -rays (12). Currently, drug resistance is a significant obstacle during the course of chemotherapy as treatment for NSCLC. This study suggests that MEX3A is a potential target for the relief of drug resistance.

In future studies, we will focus on the effect of MEX3A on the transcription of related genes. We will detect the RNA content of various genes to determine the regulatory effect of MEX3A on transcription. The histochemical results of our study suggested that MEX3A was predominately expressed in the nucleus. However, previous studies have shown that MEX3A is present in both the nucleus and the cytoplasm and can shuttle between them. We will observe the localization of MEX3A in cells in response to various treatments of cells such as transfection or U0126. This will allow us to elucidate the mechanisms behind the shuttling of MEX3A between the nucleus and cytoplasm. It is also critical to determine the mechanism by which MEX3A promotes the biological behavior of NSCLC beyond its influence on the MEK/ERK pathway. In addition, by simulating radiotherapy and chemotherapy on NSCLC cells, we can elucidate how the expression of MEX3A effects treatment resistance in NSCLC. Drug resistance is a major obstacle in tumor treatment and if we can contribute relieving this issue, we will vastly improve tumor treatment.

5 Conclusion

In summary, our study has shown that MEX3A is related to the poor prognosis of NSCLC by promoting the expression of cell proliferation proteins, and those involved in migration and invasion of cancer cells. Moreover, the expression of proteins within the MAPK pathway were enhanced by

291 MEX3A. These results indicate that MEX3A has potential as a target for future clinical treatments of
292 NSCLC.

293 **6 List of abbreviations**

294 MEX3A: MEX3 homolog A; Ago: Argonaute; CDX2: Caudal type homeobox 2; NSCLC: Non-
295 small-cell lung carcinoma; ERK: Extracellular regulated protein kinases; P-body: Processing body;
296 MEM: Modified Eagle medium; PBS: Phosphate-buffered saline; MMP: Matrix metalloproteinase;
297 siRNA: Small-interfering RNA; MAPK: Mitogen-activated protein kinase. TNM: Tumor, Node,
298 Metastasis; UICC: International Union against Cancer; WHO: World Health Organization; MRE:
299 MEX-3 recognition element.

300 **7 Declarations**

301 Ethics approval and consent to participate: This study was approved by the Medical Research Ethics
302 Committee of China Medical University and informed consent was obtained from all patients.

303 Consent for publication: not applicable

304 Availability of data and materials:

305 Datasets generated and/or analysed during the current study are available in the UNIPROT repository,
306 <https://www.uniprot.org/>

307 Competing interest: The authors declare that they have no competing interests.

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310 Authors' contributions: ZY designed the study, conducted experiments, acquired and analyzed data,
311 and wrote the manuscript. ZB, HJ, YT, JJ, WD, QF, QZ, HB, XZ, ZF and ZA conducted the
312 experiments and acquired data. XQ was responsible for the conception and supervision of the study
313 and wrote the manuscript. All authors corrected drafts and approved the final version of the
314 manuscript.

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388 **9 Figure Legends and Tables**

389 Figure 1

390

391 Expression of MEX3A in non-small cell lung cancer (NSCLC) tissues and cell lines. A. protein
 392 expression as analyzed by immunohistochemistry in (a) alveolar and (b) normal bronchial epithelial
 393 cells; (c) well-differentiated adenocarcinoma and (d) poorly-differentiated adenocarcinoma; (e) well-
 394 differentiated squamous cell carcinoma and (f) poorly-differentiated squamous cell carcinoma.
 395 Magnification: $\times 200$. B. Relative expression of MEX3A protein in eight paired NSCLC samples and
 396 adjacent normal lung tissue. C. MEX3A protein levels in seven lung cancer cell lines and a normal
 397 bronchial cell line (HBE) were assessed by immunoblotting and analyzed with ImageJ software. D.
 398 Survival of NSCLC patients with respect to MEX3A expression was analyzed with a Kaplan-Meier
 399 curve; hazard ratio (HR) and P-value are indicated.

400

401 Figure 2

402

403 Effect of MEX3A expression on colony formation and proliferation of NSCLC cells. A549 and
404 H1299 cells were transfected with MEX3A-specific siRNA (si-MEX3A) or control siRNA (control),
405 or with a MEX3A expression plasmid (MEX3A) or vehicle (empty). *P<0.05, **P<0.01 and
406 ***P<0.001. A. Colony formation assay. (a) A549: si-MEX3A vs control; MEX3A vs empty. (b)
407 H1299: si-MEX3A vs control; MEX3A vs empty. B. MTT assay. (a) A549: si-MEX3A vs control;

408 MEX3A vs empty. (b) H1299: si-MEX3A vs control; MEX3A vs empty. C, E. Changes in
 409 expression of cell proliferation-related proteins in (D) A549 and (E) H1299 cells.

410

411 Figure 3

412

413 Effect of MEX3A expression on the migration and invasion of NSCLC cells. A549 and H1299 cells
 414 were transfected with MEX3A-specific siRNA (si-MEX3A) or control siRNA (control), or with a
 415 MEX3A expression plasmid (MEX3A) or vehicle (empty). *P<0.05, **P<0.01 and ***P<0.001. A.

Cell migration as analyzed by a Transwell migration assay; cells that migrated to the lower chamber were stained with hematoxylin and counted. (a) A549 migration: si-MEX3A vs control; MEX3A vs empty. (b) H1299 migration: si-MEX3A vs control; MEX3A vs empty. (c) A549 invasion: si-MEX3A vs control; MEX3A vs empty. (d) H1299 invasion: si-MEX3A vs control; MEX3A vs empty. B,C. Expression of MEX3A and cell migration- and invasion-related proteins in transfected (B) A549 and (C) H1299 cells.

Figure 4

Changes in the expression of MAPK-related proteins in (A) A549 and (B) H1299. Relative quantification analysis was based on grayscale values. *P<0.05, **P<0.01 and ***P<0.001.

Figure 5

Effect of MEX3A with or without ERK inhibition on the behavior of NSCLC cells. A549 and H1299 cells transfected with a MEX3A expression plasmid were treated with the MEK inhibitor U0126 or DMSO (control) and analyzed for cell proliferation: (A) colony formation assay and (B) MTT assay; and (C) migration and invasion.

Table 1

Antibodies used for western blotting in the present study.

Antibody name	Source	Catalog number	Host	Dilution
MEX3A	Sigma-Aldrich	PRS4869	Rabbit	1:1000
Actin	Proteintech	600004-1-1g	Mouse	1:10000
GAPDH	Proteintech	66009-1-1g	Mouse	1:10000
CyclinA2	Cell Signaling Technology Inc.	4656	Mouse	1:1000
CDK4	Cell Signaling Technology Inc.	12790	Rabbit	1:1000
CDK6	Cell Signaling Technology Inc.	13331	Rabbit	1:1000
RhoA	Cell Signaling Technology Inc.	2117	Rabbit	1:1000
MMP2	Cell Signaling Technology Inc.	20994	Rabbit	1:1000
P-MEK	Cell Signaling Technology Inc.	9127	Rabbit	1:1000
P-ERK	Cell Signaling Technology Inc.	4370	Rabbit	1:1000
ERK	Cell Signaling Technology Inc.	4696	Mouse	1:1000

437

438 Table 2

439 Association of MEX3A expression with the clinical and pathological characteristics of NSCLC

440 patients.

Clinicopathological characteristics	Total N	MEX3A-negative	MEX3A-positive	P-value
Age (years)				
≥60	38	19	19	0.295

<60	49	19	32	
Gender				
Male	63	29	34	0.473
Female	24	9	15	
Histological type				
Squamous ccell carcinoma	47	21	26	0.838
Adenocarcinoma	40	17	23	
Differentiation				
Well-Moderate	31	18	13	0.044
Poor	56	20	29	
Tumor size (cm)				
≥ 3	47	15	32	0.02
<3	40	23	17	
Lymph node metastasis				
Negative	42	26	16	0.001
Positive	45	12	33	
TNM stage				
I	48	27	21	0.009
II-III	39	11	28	

442 Table 3

443 P value of result 3.6

colony information	MTT assay	Transwell without Matrigel	Transwell with Matrigel
A549	0.0047	0.0009	0.0076
H1299	0.0004	0.0002	0.0006

444

Figures

Figure 2

Expression of MEX3A in non-small cell lung cancer (NSCLC) tissues and cell lines. A. protein expression as analyzed by immunohistochemistry in (a) alveolar and (b) normal bronchial epithelial cells; (c) well-differentiated adenocarcinoma and (d) poorly-differentiated adenocarcinoma; (e) well differentiated squamous cell carcinoma and (f) poorly-differentiated squamous cell carcinoma. Magnification: $\times 200$. B. Relative expression of MEX3A protein in eight paired NSCLC samples and adjacent normal lung tissue. C. MEX3A protein levels in seven lung cancer cell lines and a normal bronchial cell line (HBE) were assessed by immunoblotting and analyzed with ImageJ software. D. Survival of NSCLC patients with respect to MEX3A expression was analyzed with a Kaplan-Meier curve; hazard ratio (HR) and P-value are indicated.

Figure 4

Effect of MEX3A expression on colony formation and proliferation of NSCLC cells. A549 and H1299 cells were transfected with MEX3A-specific siRNA (si-MEX3A) or control siRNA (control), or with a MEX3A expression plasmid (MEX3A) or vehicle (empty). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. A. Colony formation assay. (a) A549: si-MEX3A vs control; MEX3A vs empty. (b) H1299: si-MEX3A vs control; MEX3A vs empty. B. MTT assay. (a) A549: si-MEX3A vs control; MEX3A vs empty. (b) H1299: si-MEX3A vs control; MEX3A vs empty. C, E. Changes in expression of cell proliferation-related proteins in (D) A549 and (E) H1299 cells.

Figure 6

Effect of MEX3A expression on the migration and invasion of NSCLC cells. A549 and H1299 cells were transfected with MEX3A-specific siRNA (si-MEX3A) or control siRNA (control), or with a MEX3A expression plasmid (MEX3A) or vehicle (empty). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. A. Cell migration as analyzed by a Transwell migration assay; cells that migrated to the lower chamber were stained with hematoxylin and counted. (a) A549 migration: si-MEX3A vs control; MEX3A vs empty. (b) H1299 migration: si-MEX3A vs control; MEX3A vs empty. (c) A549 invasion: si-MEX3A vs control; MEX3A vs empty. (d) H1299 invasion: si-MEX3A vs control; MEX3A vs empty. B,C. Expression of MEX3A and cell migration- and invasion-related proteins in transfected (B) A549 and (C) H1299 cells.

Figure 8

Changes in the expression of MAPK-related proteins in (A) A549 and (B) H1299. Relative quantification analysis was based on grayscale values. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 10

Effect of MEX3A with or without ERK inhibition on the behavior of NSCLC cells. A549 and H1299 cells transfected with a MEX3A expression plasmid were treated with the MEK inhibitor U0126 or DMSO (control) and analyzed for cell proliferation: (A) colony formation assay and (B) MTT assay; and (C) migration and invasion.

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