

Druggable Oncogene Fusions in Invasive Mucinous Lung Adenocarcinoma

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Abstract

Purpose: To identify druggable oncogenic fusions in invasive mucinous adenocarcinoma (IMA) of the lung, a malignant type of lung adenocarcinoma in which *KRAS* mutations frequently occur.

Experimental Design: From an IMA cohort of 90 cases, consisting of 56 cases (62%) with *KRAS* mutations and 34 cases without (38%), we conducted whole-transcriptome sequencing of 32 IMAs, including 27 cases without *KRAS* mutations. We used the sequencing data to identify gene fusions, and then performed functional analyses of the fusion gene products.

Results: We identified oncogenic fusions that occurred mutually exclusively with *KRAS* mutations: *CD74-NRG1*, *SLC3A2-NRG1*, *EZR-ERBB4*, *TRIM24-BRAF*, and *KIAA1468-RET*. *NRG1* fusions were present in 17.6% (6/34) of *KRAS*-negative IMAs. The *CD74-NRG1* fusion activated HER2:HER3 signaling, whereas the *EZR-ERBB4* and *TRIM24-BRAF* fusions constitutively activated the ERBB4 and BRAF kinases, respectively. Signaling pathway activation and fusion-induced anchorage-independent growth/tumorigenicity of NIH3T3 cells expressing these fusions were suppressed by tyrosine kinase inhibitors approved for clinical use.

Conclusions: Oncogenic fusions act as driver mutations in IMAs without *KRAS* mutations, and thus represent promising therapeutic targets for the treatment of such IMAs. *Clin Cancer Res*; 20(12); 3087–93. ©2014 AACR.

Introduction

Oncogene fusions have recently been identified as driver mutations and (possible) therapeutic targets in lung adenocarcinoma (LADC), a major histologic type of lung cancer (1). Such fusions include *MLL4- or KIF5B-ALK*, *KIF5B*, or *CCDC6-RET*, and *CD74-*, *EZR-*, or *SLC3A2-*

ROS1 (2–9). These oncogene fusions occur mutually exclusively with one another, and with other targetable oncogene aberrations such as *EGFR*, *KRAS*, *BRAF*, and *HER2* mutations. Therefore, molecular targeted therapy combined with the identification of driver oncogene aberrations represents a powerful and promising approach to personalized treatment of LADC (10, 11).

Invasive mucinous adenocarcinoma (IMA) of the lung is composed predominantly of goblet cells. IMA is morphologically characterized by tall columnar cells with basal nuclei and a pale cytoplasm containing varying amounts of mucin (12, 13). IMAs, which constitute 2% to 10% of all LADCs in Japan, the United States, and European countries (14–16), are indicated as being more malignant than more common types of LADC, such as acinar or papillary adenocarcinoma. The *KRAS* mutation is the only driver aberration commonly detected in IMAs (in 50%–80% of cases). To date, no driver gene aberrations have been detected in *KRAS*-negative IMAs; these aberrations must be identified to facilitate the development of effective treatments for such cancers. Therefore, we performed whole-transcriptome sequencing (RNA sequencing) of IMAs lacking *KRAS* mutations to identify novel chimeric fusion transcripts that represent potential targets for cancer therapy.

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Translational Relevance

Oncogene fusions, such as the *ALK*, *RET*, and *ROS1* fusions, have recently been revealed as therapeutic targets in lung adenocarcinoma (LDAC). We identified multiple druggable oncogene fusions, including those involving the *NRG1*, *ERBB4*, and *BRAF* genes, in invasive mucinous adenocarcinoma (IMA), a malignant type of LDAC. The fusions occurred mutually exclusively with *KRAS* mutations, a common driver oncogene aberration in IMA. These fusions represent potentially clinically relevant targets for treatment of IMAs that lack *KRAS* mutations.

Materials and Methods

Samples

Ninety IMAs were identified among consecutive patients with primary adenocarcinoma of the lung who were treated surgically at the National Cancer Center Hospital, Tokyo, Japan, from 1998 to 2013. Histologic diagnoses were based on the most recent World Health Organization classification and the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) criteria for LADC (13, 17). Total RNA was extracted from grossly dissected, snap-frozen tissue samples using TRIzol (Invitrogen). The study was approved by the Institutional Review Boards of the participating institutions.

RNA sequencing

RNA sequencing libraries were prepared from 1 or 2 µg of total RNA using the mRNA-Seq Sample Prep Kit or TruSeq RNA Sample Prep Kit (Illumina). The resultant libraries were subjected to paired-end sequencing of 50 or 75 bp reads on a Genome Analyzer IIx (GAIIx) or HiSeq 2000 (Illumina). Fusion transcripts were detected using the TopHat-Fusion algorithm (18). Experimental conditions for RNA sequencing are described in Supplementary Table S1.

Examinations of oncogenic properties of fusion products

To construct lentiviral vectors for expression of the CD74-*NRG1*, *EZR-ERBB4*, and *TRIM24-BRAF* fusion proteins, full-length cDNAs were amplified from tumor cDNA by PCR and inserted into pLenti-6/V5-DEST plasmids (Invitrogen). The integrity of each inserted cDNA was verified by Sanger sequencing. Expression of fusion products of the predicted sizes was confirmed by Western blot analysis of transiently transfected and virally infected cells (Supplementary Fig. S1A). Details of plasmid transfection, viral infection, Western blot analysis, and soft agar colony and tumorigenicity assays are described in Supplementary Materials and Methods.

Results and Discussion

We prepared an IMA cohort of 90 cases consisting of 56 (62%) cases with *KRAS* mutations and 34 (38%) cases without. The 34 *KRAS*-negative cases included two, one, and one cases with *BRAF* mutation, *EGFR* mutation, and *EML4-ALK* fusion, respectively; the remaining 30 were "pan negative" for representative driver aberrations in LADCs. Thirty-two cases, consisting of 27 pan-negative and five *KRAS* mutation-positive cases, were subjected to RNA sequencing (Supplementary Table S1). Analysis of $>2 \times 10^7$ paired-end reads obtained by RNA sequencing and subsequent validation by Sanger sequencing of reverse transcription PCR (RT-PCR) products revealed five novel gene-fusion transcripts detected only in the pan-negative IMAs: *CD74-NRG1*, *SLC3A2-NRG1*, *EZR-ERBB4*, *TRIM24-BRAF*, and *KIAA1468-RET* (Fig. 1A and B; Table 1; details in Supplementary Materials and Methods; Supplementary Fig. S2 and Supplementary Table S2). RT-PCR screening of these fusions in the remaining 58 IMAs that had not been subjected to RNA sequencing revealed one additional pan-negative case with the *CD74-NRG1* fusion. Thus, the *CD74-NRG1* fusion, detected in five of 34 (14.7%) cases negative for *KRAS* mutations, was the most frequent fusion among *KRAS* mutation-negative IMAs. Fusions of *CD74* or *SLC3A2* with *NRG1* were present in 17.6% (6/34) of cases. The five novel fusions were mutually exclusively with one another and were not present in any of the *KRAS* mutation-positive cases (Table 2).

Four of the novel fusions, *CD74-NRG1*, *SLC3A2-NRG1*, *EZR-ERBB4*, and *TRIM24-BRAF*, involved rearrangements of genes encoding protein kinases or a ligand of a receptor protein kinase (*NRG1*/neuregulin/herectin) for which oncogenic rearrangements have not been previously reported in lung cancer (Supplementary Fig. S3). The remaining fusion was a novel type involving the *RET* oncogene; fusions with *RET* are observed in 1% to 2% of LADCs (4, 5, 7, 8, 11). In a screen of 315 LADCs without IMA features from Japanese patients and 144 consecutive LADCs from U.S. patients, all tumors were negative for all of the *NRG1*, *BRAF*, and *ERBB4* fusions, as well as the novel *RET* fusion. Therefore, these fusions might be driver aberrations specific to LADCs with IMA features. The four novel gene fusions were likely to have been caused by interchromosomal translocations or paracentric inversion (Table 1 and Supplementary Fig. S3). Consistently, separation of the signals generated by the probes flanking the translocation sites of *NRG1* in fusion-positive tumors was observed upon FISH analysis of *CD74-NRG1* fusion-positive tumors (Supplementary Fig. S4). We also confirmed overexpression of *NRG1*, *ERBB4*, and *BRAF* proteins in tumor cells carrying the corresponding fusions by immunohistochemical analysis, using antibodies recognizing polypeptides retained in the fusion proteins; expression of *NRG1*, *ERBB4*, and *BRAF* proteins was also observed in some fusion-negative cases (Supplementary Fig. S5). IMAs harboring gene fusions were obtained from both male and female patients, although

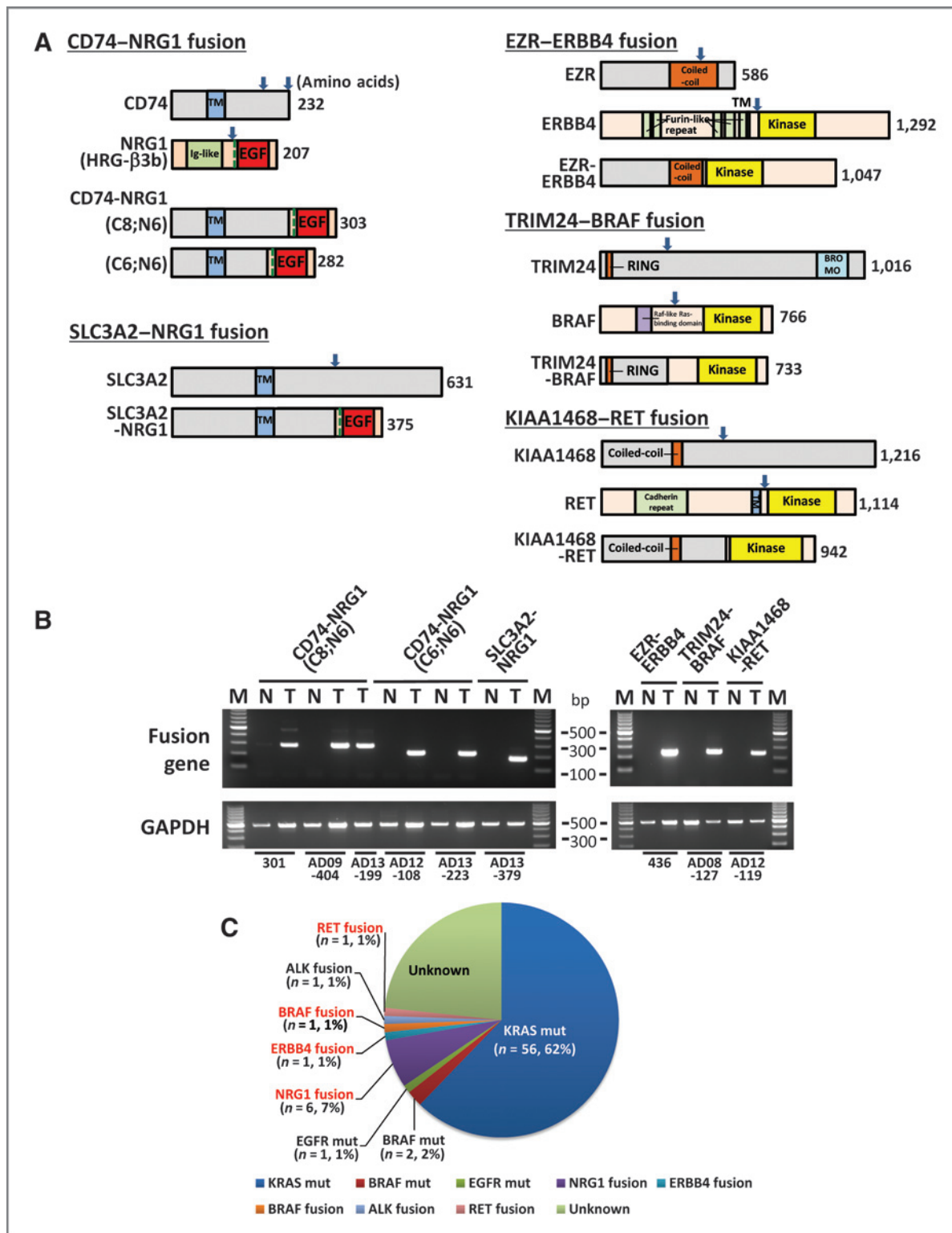


Figure 1. Oncogenic fusions in invasive mucinous LDAC. **A**, schematic representations of the wild-type proteins (top rows of each section) followed by the fusion proteins identified in this study. The breakpoints for each variant are indicated by blue arrows. TM, transmembrane domain. Locations of putative cleavage sites in the NRG1 polypeptide are indicated by dashed green lines. **B**, detection of gene-fusion transcripts by RT-PCR. RT-PCR products for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown below. Six IMAs (T) positive for gene fusions are shown alongside their corresponding non-cancerous lung tissues (N); labels below the gel image indicate sample IDs (see Table 1). **C**, pie chart showing the fraction of IMAs that harbor the indicated driver mutations.

Table 1. Characteristics of invasive mucinous LDACs with novel gene fusions

No.	Sample	Sex	Age	Smoking (pack/year)	Gene fusion	Chromosome aberration	Oncogene mutation ^a	Pathologic stage	TTF1	HNF4A
1	301T	M	55	Ever (47)	<i>CD74-NRG1</i>	t(5;8)(q32;p12)	None	1a	–	+
2	AD12-108T	F	68	Never	<i>CD74-NRG1</i>		None	2b	–	+
3	AD09-404T	F	78	Never	<i>CD74-NRG1</i>		None	1a	–	+
4	AD13-199T	F	47	Never	<i>CD74-NRG1</i>		None	1b	–	+
5	AD13-223T	F	53	Never	<i>CD74-NRG1</i>		None	1a	–	+
6	AD13-379T	F	66	Never	<i>SLC3A2-NRG1</i>	t(8;11)(p12;q13)	None	1b	Not tested	Not tested
7	436T	M	61	Ever (41)	<i>EZR-ERBB4</i>	t(2;6)(q25;q34)	None	1b	–	+
8	AD08_127T	F	66	Never	<i>TRIM24-BRAF</i>	inv7(q33;q34)	None	1a	+	+
9	AD12-119T	M	62	Current (63)	<i>KIAA1468-RET</i>	t(10;18)(q21;q11)	None	1a	+	–

^aEGFR, KRAS, BRAF, and HER2 mutations and ALK, RET, and ROS1 fusions.

NRG1 fusion-positive cases were preferentially from female never smokers (Table 1).

The CD74-*NRG1* and SLC3A2-*NRG1* fusion proteins, whose sequences were deduced from RNA sequencing data, contained the CD74 or SLC3A2 transmembrane domain and retained the EGF-like domain of the *NRG1* protein (*NRG1* III-β3 form; Fig. 1A). The *NRG1* III-β3 protein has a cytosolic N-terminus and a membrane-tethered EGF-like domain, and mediates juxtacrine signals signaling through HER2:HER3 receptors (19). Because parts of CD74 or SLC3A2 replaced the transmembrane domain of wild-type *NRG1* III-β3, we speculated that the membrane-tethered EGF-like domain might activate juxtacrine signaling through HER2:HER3 receptors. In addition, it was also possible that expression of these fusion proteins resulted in the production of soluble *NRG1* protein due to proteolytic cleavage at sites derived from *NRG1* (dashed green lines in Fig. 1A), as recently suggested for *NRG1* type III proteins (20, 21). Exposing EFM-19 cells to conditioned media from H1299 human lung cancer cells expressing exogenous CD74-*NRG1* fusion protein resulted in phosphorylation of endogenous ERBB2/HER2 and ERBB3/HER3 proteins, suggesting that autocrine HER2:HER3 sig-

naling was activated by secreted *NRG1* ligands generated from CD74-*NRG1* polypeptides (Fig. 2A). Phosphorylation of extracellular signal-regulated kinase (ERK) and AKT, downstream mediators of HER2:HER3, was also elevated. HER2, HER3, and ERK phosphorylation was suppressed by lapatinib and afatinib, U.S. Food and Drug Administration (FDA)-approved tyrosine kinase inhibitors (TKI) that target HER kinases (22–24). Together, these observations indicate that *NRG1* fusions activated HER2:HER3 signaling by juxtacrine and/or autocrine mechanisms.

The *EZR-ERBB4* fusion protein contained the *EZR* coiled-coil domain, which functions in protein dimerization, and also retained the full *ERBB4* kinase domain (Fig. 1A). These features indicated that the *EZR-ERBB4* protein is likely to form a homodimer via the coiled-coil domain of *EZR*, causing aberrant activation of the kinase function of *ERBB4*, similar to the situation of *EZR-ROS1* fusion (5). Indeed, when the *EZR-ERBB4* cDNA was exogenously expressed in NIH3T3 fibroblasts, tyrosine 1258, located in the activation loop of the *ERBB4* kinase site, was phosphorylated in the absence of serum stimulation, indicating that fusion with *EZR* aberrantly activated the *ERBB4* kinase (Fig. 2B). Consistent with this, phosphorylation of a downstream

Table 2. Characteristics of 90 invasive mucinous LDACs

Variable	All	Mutation				Fusion					None (%)
		KRAS	BRAF	EGFR	CD74-NRG1 or EZR-SLC3A2-NRG1	TRIM24-ERBB4	EML4-BRAF	KIAA1468-ALK	RET		
Total	90 (100)	56 (62.2)	2 (2.2)	1 (1.1)	6 (6.7)	1 (1.1)	1 (1.1)	1 (1.1)	1 (1.1)	21 (23.3)	
Age (mean ± SD; y)	67.2 ± 9.7	68.1 ± 9.7	66.5 ± 3.5	50	61.2 ± 11.5	61	66	64	62	68.1 ± 9.6	
Sex											
Male (%)	39 (43.3)	28 (50.0)	0 (0)	0 (0)	1 (16.7)	1 (100)	0 (0)	0 (0)	1 (100)	8 (38.1)	
Female (%)	51 (56.7)	28 (50.0)	2 (100)	1 (100)	5 (83.3)	0 (0)	1 (100)	1 (100)	0 (0)	13 (61.9)	
Smoking habit											
Never smoker (%)	51 (56.7)	29 (51.8)	2 (100)	1 (100)	4 (66.7)	0 (0)	1 (100)	1 (100)	0 (0)	13 (61.9)	
Ever smoker (%)	39 (43.3)	27 (48.2)	0 (0)	0 (0)	2 (33.3)	1 (100)	0 (0)	0 (0)	1 (100)	8 (38.1)	

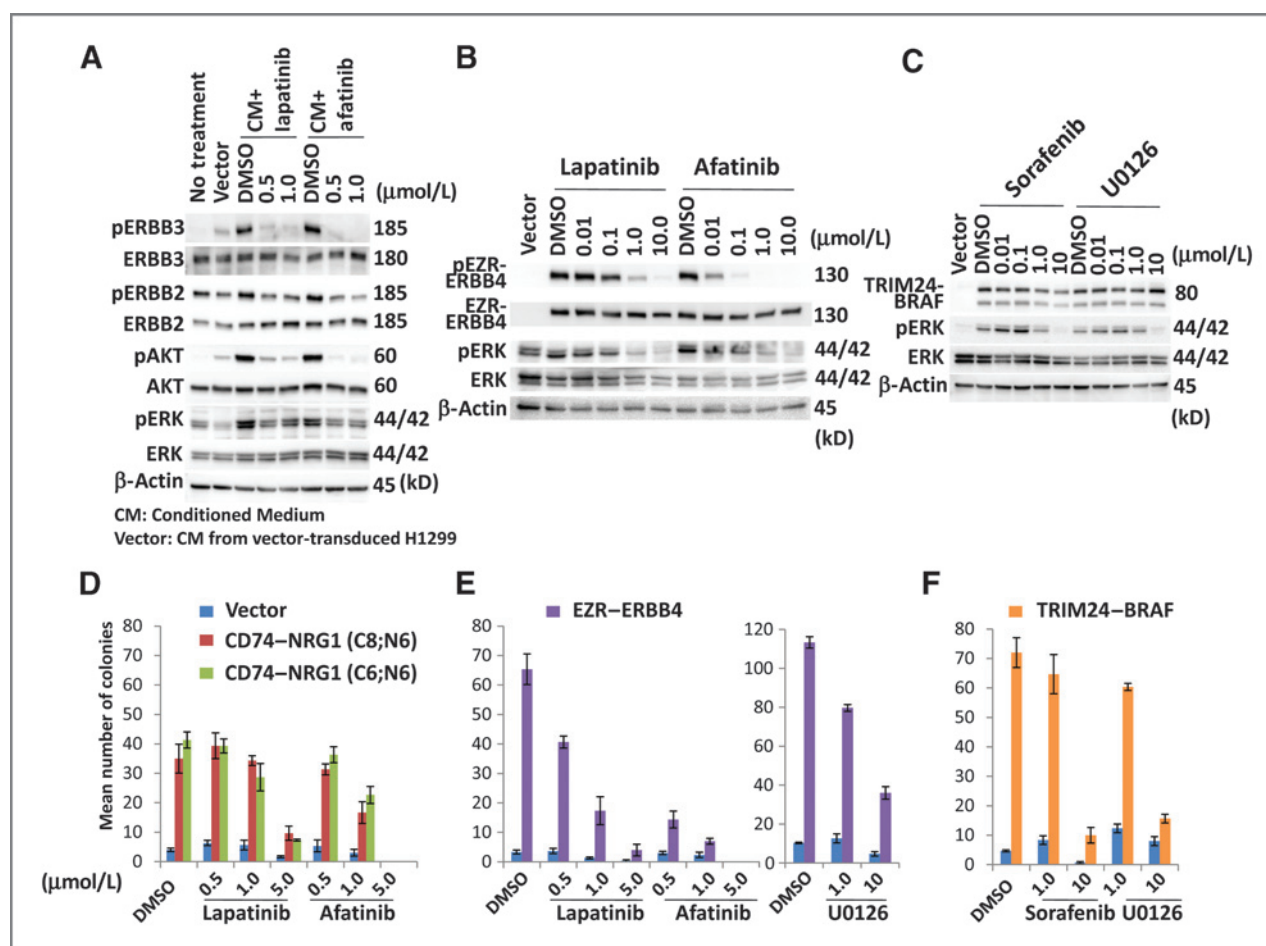


Figure 2. Oncogenic properties of gene-fusion products. **A**, ERBB3 activation by *CD74-NRG1* fusion, demonstrated using the EFM-19 cell system. ERBB3, ERBB2, AKT, and ERK phosphorylation were examined in EFM-19 (reporter) cells treated for 30 minutes with conditioned media from H1299 cells exogenously expressing *CD74-NRG1* cDNA. Phosphorylation was suppressed by HER-TKIs. **B**, ERBB4 activation by *EZR-ERBB4* fusion. Stably transduced NIH3T3 cells were serum-starved for 24 hours and treated for 2 hours with DMSO (vehicle control) or TKIs. Phosphorylation of ERBB4 and ERK was suppressed by ERBB4-TKIs. EZR-ERBB4 protein was detected using an antibody recognizing ERBB4 polypeptides retained in the fusion protein. **C**, BRAF activation by *TRIM24-BRAF* fusion. Stably transduced NIH3T3 cells were serum-starved for 24 hours and treated for 2 hours with DMSO or kinase inhibitors. ERK phosphorylation (activation) was suppressed by sorafenib, a kinase inhibitor targeting BRAF, as well as by U0126, a MEK inhibitor. TRIM24-BRAF protein was detected using an antibody recognizing BRAF polypeptides retained in the fusion protein. **D–F**, anchorage-independent growth of NIH3T3 cells expressing *CD74-NRG1* (**D**), *EZR-ERBB4* (**E**), or *TRIM24-BRAF* (**F**) cDNA, and suppression of this growth by kinase inhibitors. Mock-, *CD74-NRG1*-, *EZR-ERBB4*-, and *TRIM24-BRAF*-transduced NIH3T3 cells were seeded in soft agar with DMSO alone or kinase inhibitors. Colonies > 100 μm in diameter were counted after 14 days. Column graphs show mean numbers of colonies $\pm$ SEM.

mediator ERK was also elevated. Phosphorylation of ERBB4 and ERK was suppressed by lapatinib and afatinib, which inhibit ERBB4 protein (22–24).

The TRIM24-BRAF fusion protein retained the BRAF kinase domain but lacked the N-terminal RAS-binding domain responsible for negatively regulating BRAF kinase. These features suggested that the fusion was constitutively active, as in the cases of the *ESRP1-BRAF* and *AGTRAP-BRAF* fusions in other cancers (25). When the *TRIM24-BRAF* cDNA was exogenously expressed in NIH3T3 cells, ERK, a downstream mediator of BRAF, was phosphorylated in the absence of serum stimulation, indicating that fusion with TRIM24 aberrantly activated BRAF kinase (Fig. 2C). ERK phosphorylation was suppressed by sorafenib, an FDA-approved drug originally

identified as a RAF kinase inhibitor (26), and also by the MEK inhibitor U0126 (Fig. 2C).

Exogenous expression of fusion gene cDNAs induced anchorage-independent growth of NIH3T3 fibroblasts, indicating their transforming activities (Fig. 2D–F). This growth was suppressed by the kinase inhibitors that suppressed fusion-induced activation of signal transduction, as described above. NIH3T3 cells expressing *EZR-ERBB4* or *TRIM24-BRAF* fusion cDNA formed tumors in nude mice (Fig. 3). Therefore, we concluded that these three fusions function as driver mutations in IMA development. We screened 200 commonly used human lung cancer cell lines, but all were negative for these three fusions (data not shown); thus, the oncogenic properties of these fusions remain unvalidated in human cancer cells.

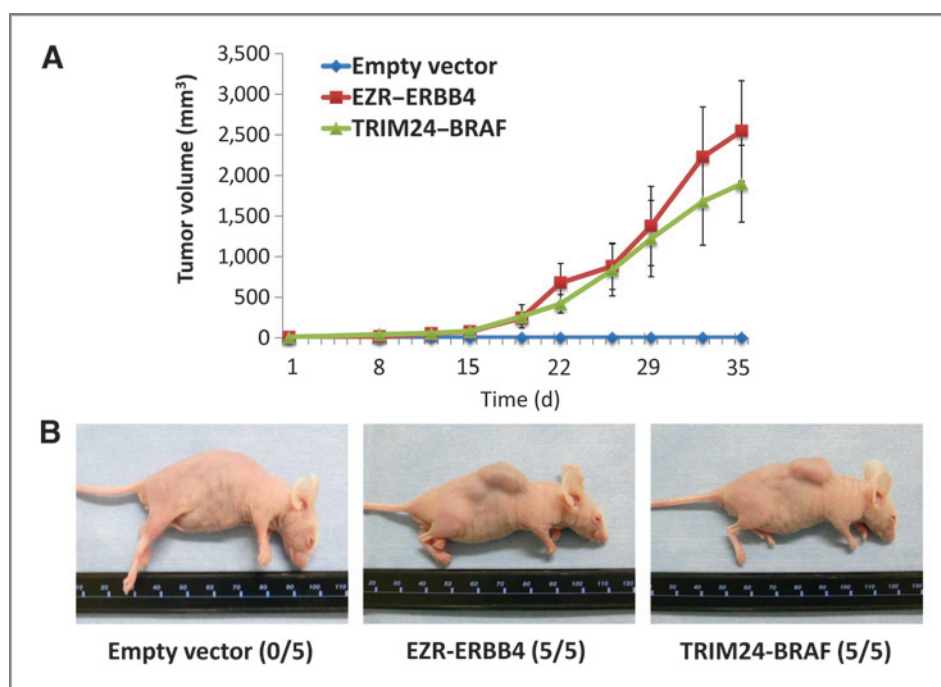


Figure 3. Tumorigenicity of NIH3T3 cells expressing *ERZ-ERBB4* or *TRIM24-BRAF* fusion cDNAs. **A**, tumor growth in nude mice injected with NIH3T3 cells expressing empty vector, *EZR-ERBB4* fusion, or *TRIM24-BRAF* fusion. Cells were resuspended with 50% Matrigel and injected into the right flank of nude mice. Tumor size was measured twice weekly for 5 weeks. Data are shown as mean $\pm$ SEM. **B**, representative tumors were photographed on day 21. The numbers in parentheses indicate the ratio of the number of mice with tumors to the number of mice receiving cell injection.

The results here suggest that the *NRG1*, *ERBB4* and *BRAF* fusions are novel driver mutations involved in the development of IMAs of the lungs (Fig. 1C) and potential targets for existing TKIs. The recurrent *NRG1* fusions were especially notable because *NRG1* was previously identified as a regulator of goblet-cell formation in primary cultures of human bronchial epithelial cells (27); therefore, activation of the *NRG1*-mediated signaling pathway (s) might play a part in IMA development by contributing to both cell transformation and acquisition of goblet-cell morphology. In addition to a small fraction of known druggable aberrations (an *ALK* fusion and an *EGFR* mutation), more than 10% (11/90; 12.2%) of IMAs harbored other druggable aberrations targeted by existing kinase inhibitors: these aberrations were represented by fusions involving *NRG1*, *ERBB4*, *BRAF*, or *RET*, or *BRAF* mutations (Table 2, Fig. 1C). To facilitate translation of these findings to the cancer clinic, it will be necessary to establish diagnostic methods, particularly using breakpoint and fusion FISH methods, capable of detecting these aberrations. Such methods will also help identify additional fusions involving other partner genes and contribute to a greater understanding of the significance of gene fusions in lung carcinogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

- Pao W, Hutchinson KE. Chipping away at the lung cancer genome. *Nat Med* 2012;18:349–51.
- Shaw AT, Engelman JA. *ALK* in lung cancer: past, present, and future. *J Clin Oncol* 2013;31:1105–11.
- Gautschi O, Zander T, Keller FA, Strobel K, Hirschmann A, Aebi S, et al. A patient with lung adenocarcinoma and *RET* fusion treated with vandetanib. *J Thorac Oncol* 2013;8:e43–4.

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4. Drilon A, Wang L, Hasanovic A, Suehara Y, Lipson D, Stephens P, et al. Response to Cabozantinib in Patients with RET Fusion-Positive Lung Adenocarcinomas. *Cancer Discov* 2013;3:630–5.
5. Takeuchi K, Soda M, Togashi Y, Suzuki R, Sakata S, Hatano S, et al. RET, ROS1 and ALK fusions in lung cancer. *Nat Med* 2012;18:378–81.
6. Mano H. ALKoma: a cancer subtype with a shared target. *Cancer Discov* 2012;2:495–502.
7. Lipson D, Capelletti M, Yelensky R, Otto G, Parker A, Jarosz M, et al. Identification of new ALK and RET gene fusions from colorectal and lung cancer biopsies. *Nat Med* 2012;18:382–4.
8. Kohno T, Ichikawa H, Totoki Y, Yasuda K, Hiramoto M, Nammo T, et al. KIF5B-RET fusions in lung adenocarcinoma. *Nat Med* 2012;18:375–7.
9. Ju YS, Lee WC, Shin JY, Lee S, Bleazard T, Won JK, et al. A transforming KIF5B and RET gene fusion in lung adenocarcinoma revealed from whole-genome and transcriptome sequencing. *Genome Res* 2012;22:436–45.
10. Oxnard GR, Binder A, Janne PA. New targetable oncogenes in non-small-cell lung cancer. *J Clin Oncol* 2013;31:1097–104.
11. Kohno T, Tsuta K, Tsuchihara K, Nakaoku T, Yoh K, Goto K. RET fusion gene: Translation to personalized lung cancer therapy. *Cancer Sci* 2013;104:1396–400.
12. Travis WD, Brambilla E, Riely GJ. New pathologic classification of lung cancer: relevance for clinical practice and clinical trials. *J Clin Oncol* 2013;31:992–1001.
13. Travis WD, Brambilla E, Noguchi M, Nicholson AG, Geisinger KR, Yatabe Y, et al. International association for the study of lung cancer/american thoracic society/european respiratory society international multidisciplinary classification of lung adenocarcinoma. *J Thorac Oncol* 2011;6:244–85.
14. Tsuta K, Kawago M, Inoue E, Yoshida A, Takahashi F, Sakurai H, et al. The utility of the proposed IASLC/ATS/ERS lung adenocarcinoma subtypes for disease prognosis and correlation of driver gene alterations. *Lung Cancer* 2013;81:371–6.
15. Warth A, Muley T, Meister M, Stenzinger A, Thomas M, Schirmacher P, et al. The novel histologic International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society classification system of lung adenocarcinoma is a stage-independent predictor of survival. *J Clin Oncol* 2012;30:1438–46.
16. Yoshizawa A, Motoi N, Riely GJ, Sima CS, Gerald WL, Kris MG, et al. Impact of proposed IASLC/ATS/ERS classification of lung adenocarcinoma: prognostic subgroups and implications for further revision of staging based on analysis of 514 stage I cases. *Mod Pathol* 2011;24:653–64.
17. Travis WD, Brambilla E, Muller-Hermelink HK, Harris CC, editors. World Health Organization classification of tumors: Pathology and genetics, tumours of lung, pleura, thymus and heart. Lyon, France: IARC Press; 2004.
18. Kim D, Salzberg SL. TopHat-Fusion: an algorithm for discovery of novel fusion transcripts. *Genome Biol* 2011;12:R72.
19. Falls DL. Neuregulins: functions, forms, and signaling strategies. *Exp Cell Res* 2003;284:14–30.
20. Fleck D, van Bebber F, Colombo A, Galante C, Schwenk BM, Rabe L, et al. Dual cleavage of neuregulin 1 type III by BACE1 and ADAM17 liberates its EGF-like domain and allows paracrine signaling. *J Neurosci* 2013;33:7856–69.
21. Dislich B, Lichtenthaler SF. The membrane-bound aspartyl protease BACE1: molecular and functional properties in Alzheimer's disease and beyond. *Front Physiol* 2012;3:8.
22. Majem M, Pallares C. An update on molecularly targeted therapies in second- and third-line treatment in non-small cell lung cancer: focus on EGFR inhibitors and anti-angiogenic agents. *Clin Transl Oncol* 2013;15:343–57.
23. Perez EA, Spano JP. Current and emerging targeted therapies for metastatic breast cancer. *Cancer* 2012;118:3014–25.
24. Nelson V, Ziehr J, Agulnik M, Johnson M. Afatinib: emerging next-generation tyrosine kinase inhibitor for NSCLC. *Onco Targets Ther* 2013;6:135–43.
25. Palanisamy N, Ateeq B, Kalyana-Sundaram S, Pflueger D, Ramnarayanan K, Shankar S, et al. Rearrangements of the RAF kinase pathway in prostate cancer, gastric cancer and melanoma. *Nat Med* 2010;16:793–8.
26. Wilhelm SM, Adnane L, Newell P, Villanueva A, Llovet JM, Lynch M. Preclinical overview of sorafenib, a multikinase inhibitor that targets both Raf and VEGF and PDGF receptor tyrosine kinase signaling. *Mol Cancer Ther* 2008;7:3129–40.
27. Kettle R, Simmons J, Schindler F, Jones P, Dicker T, Dubois G, et al. Regulation of neuregulin 1beta1-induced MUC5AC and MUC5B expression in human airway epithelium. *Am J Respir Cell Mol Biol* 2010;42:472–81.

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Druggable Oncogene Fusions in Invasive Mucinous Lung Adenocarcinoma

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Figure S1.

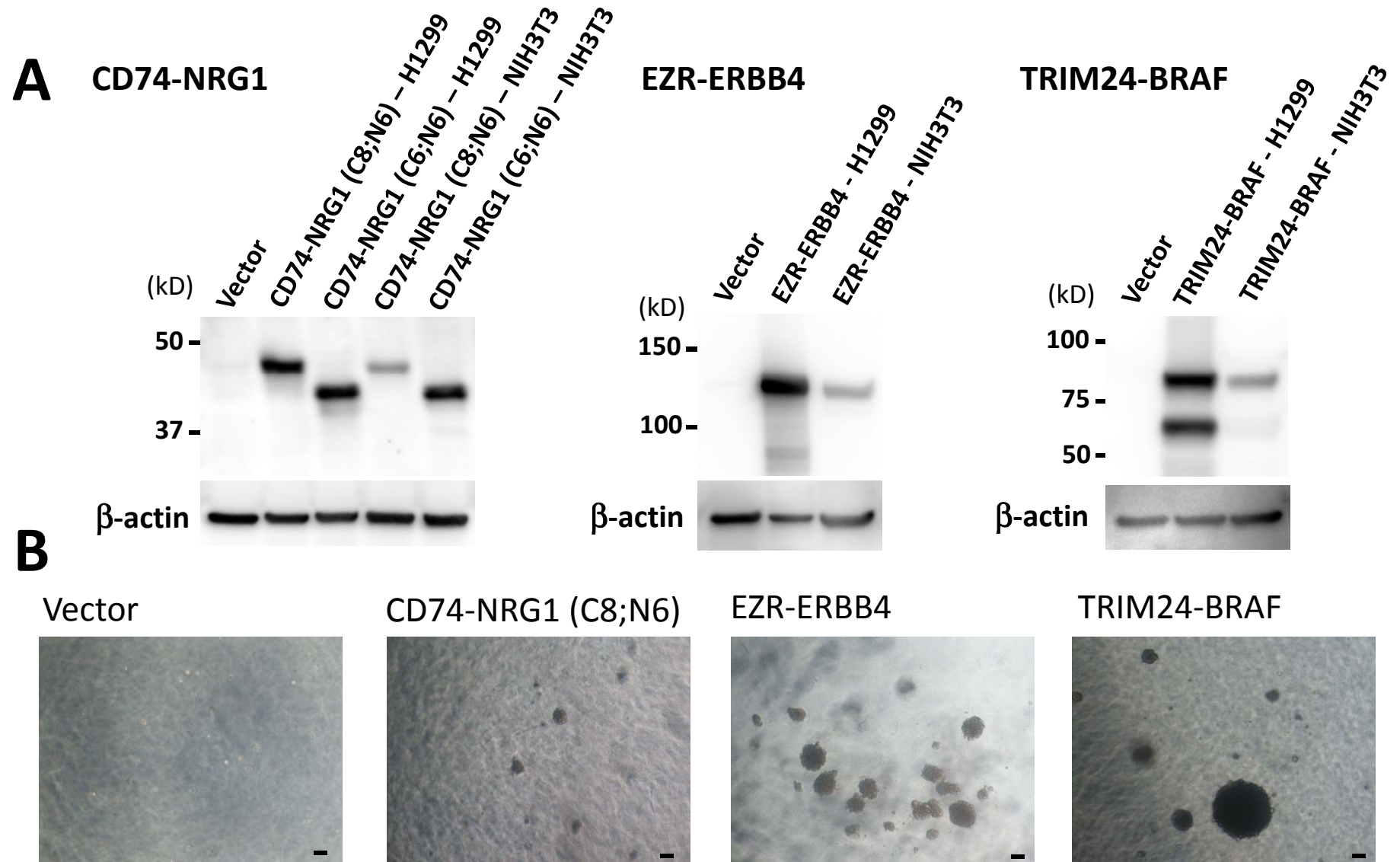


Figure S2.

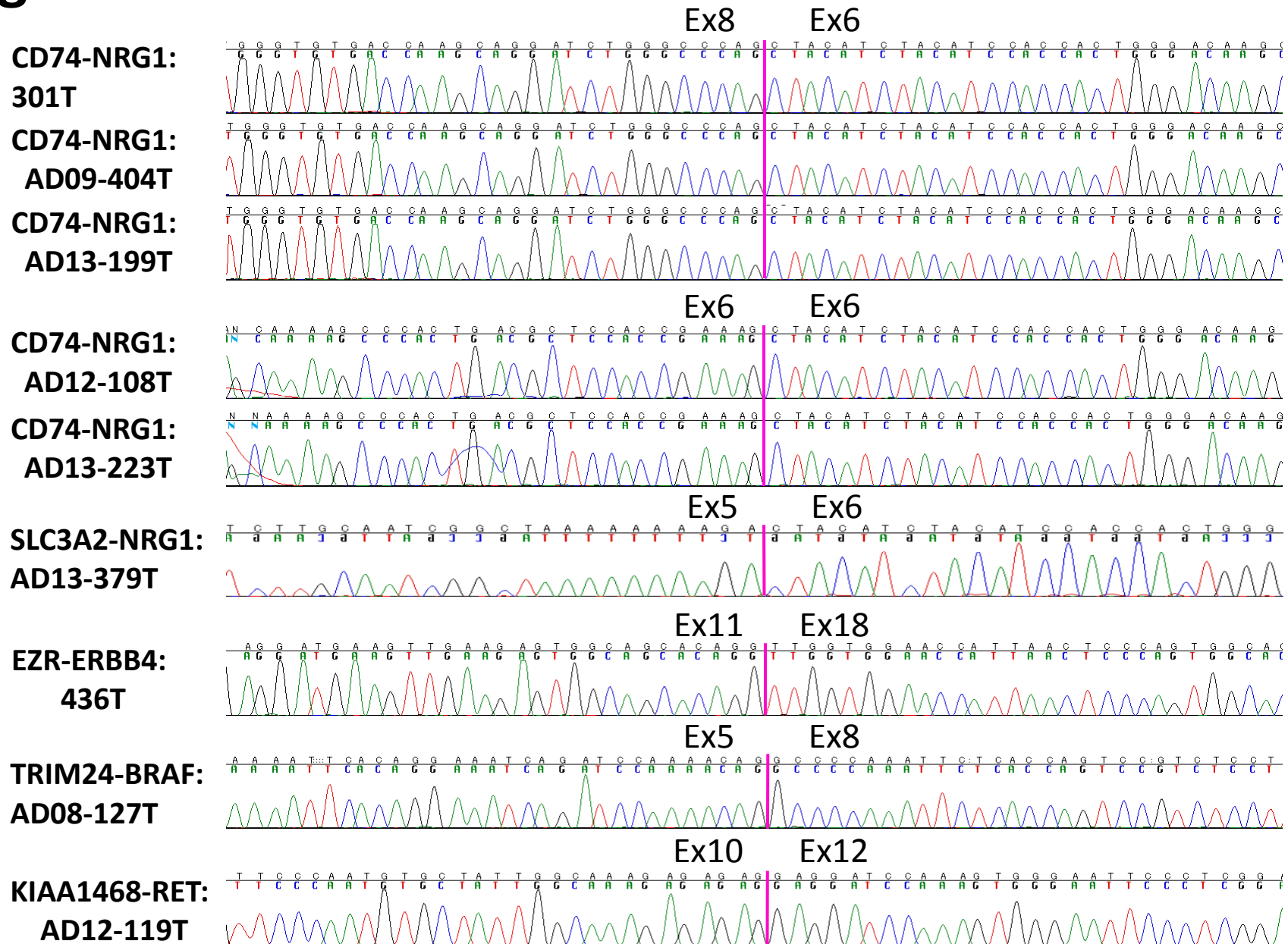
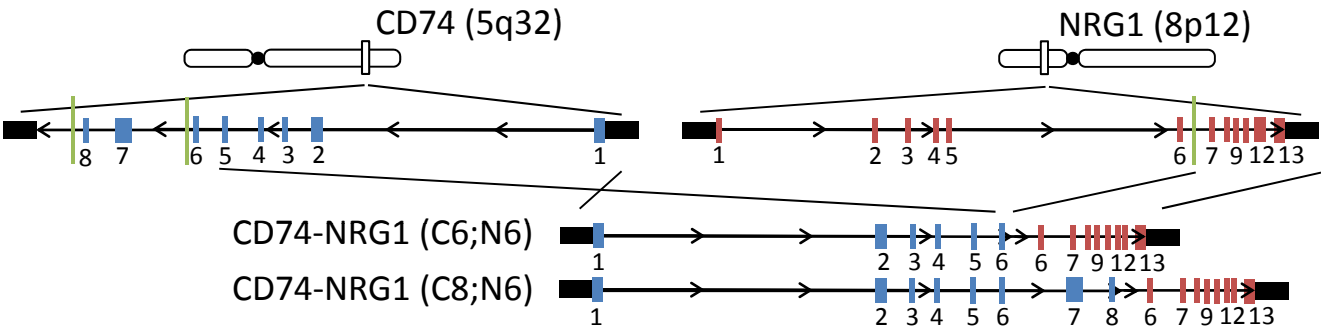


Figure S3.

A

CD74-NRG1



B

SLC3A2-NRG1

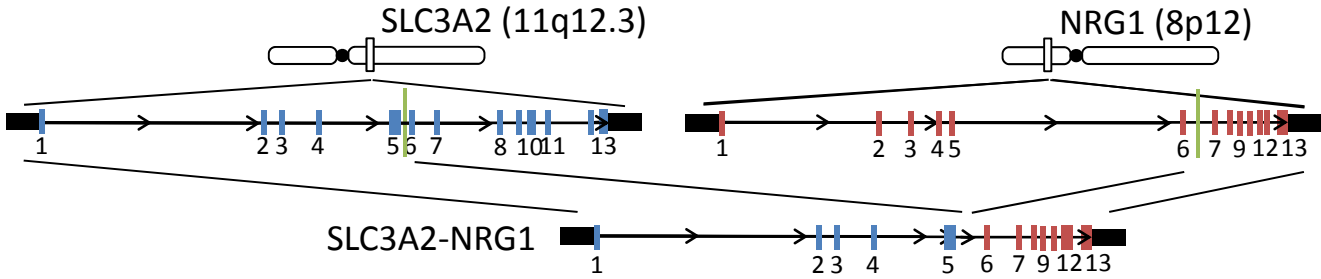
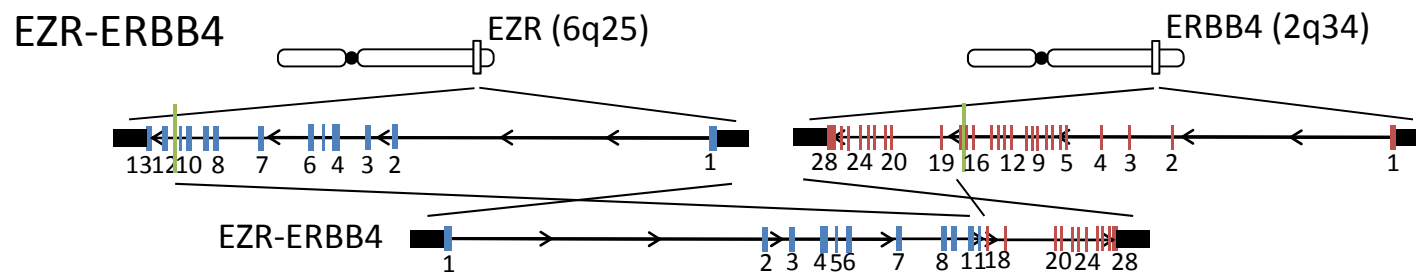


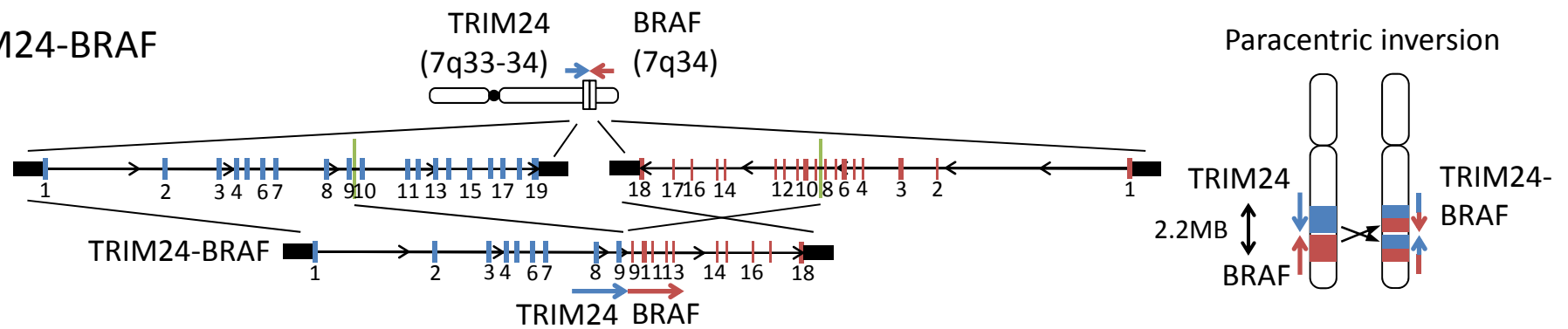
Figure S3.

C



D

TRIM24-BRAF



E

KIAA1468-RET

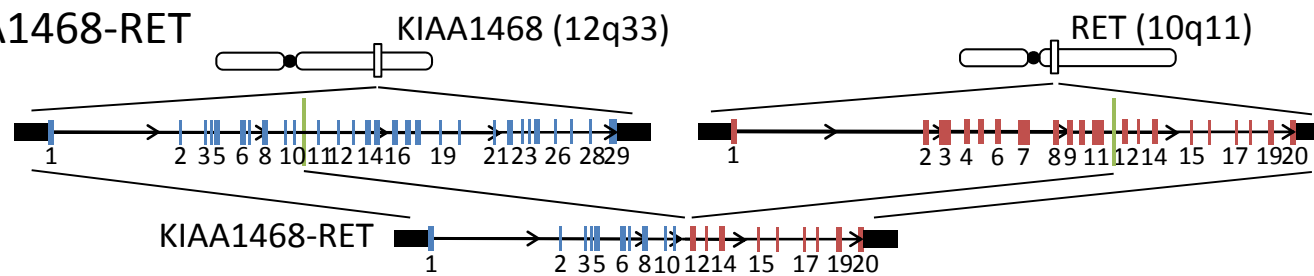
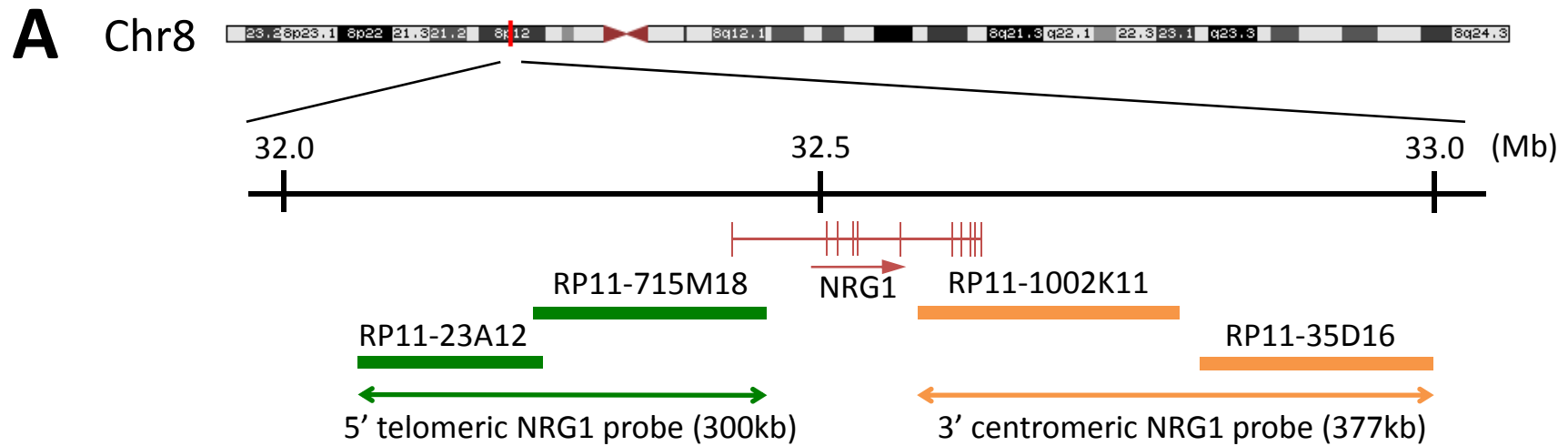
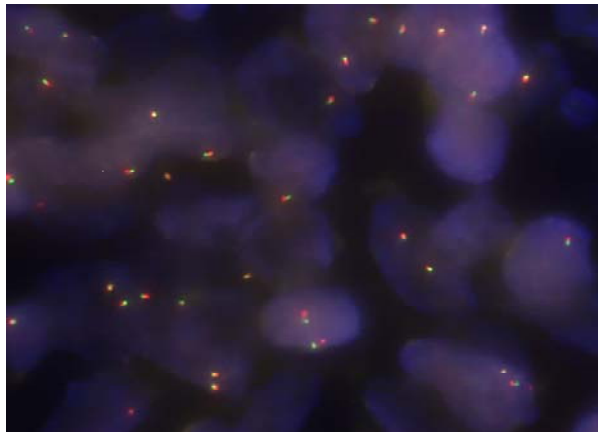


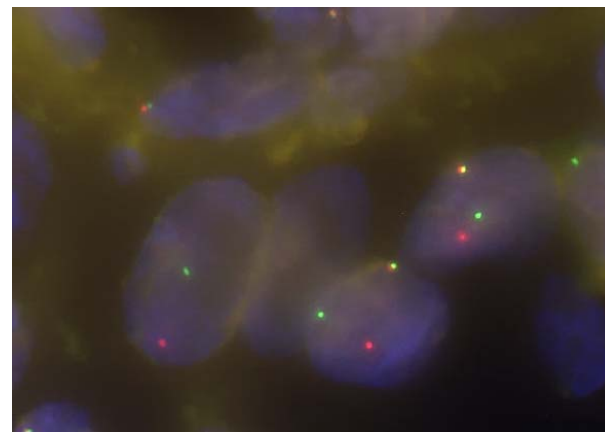
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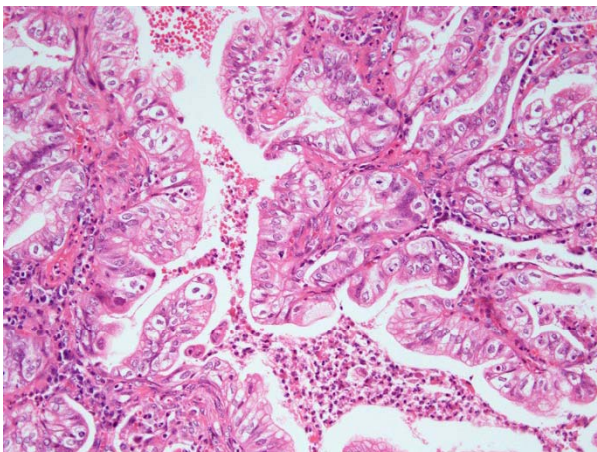
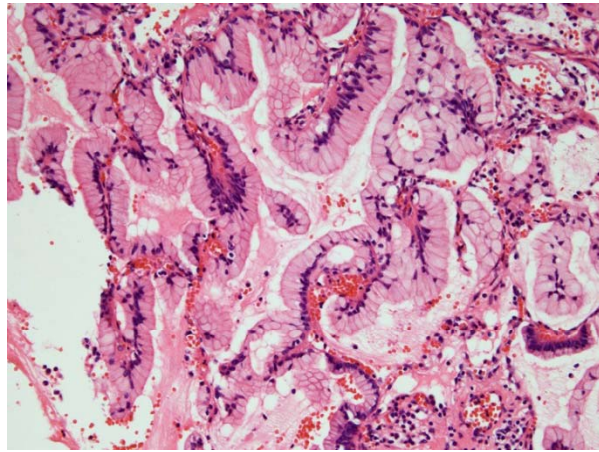
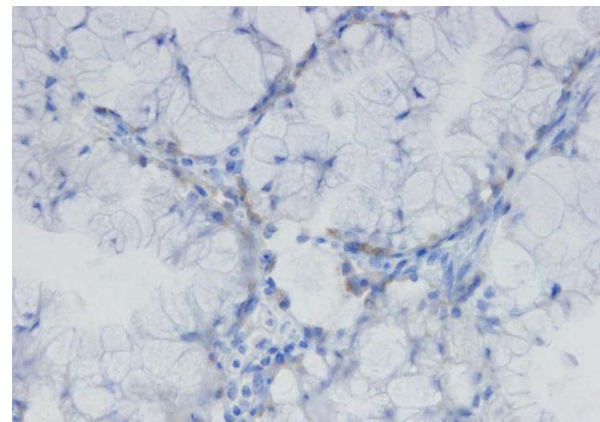
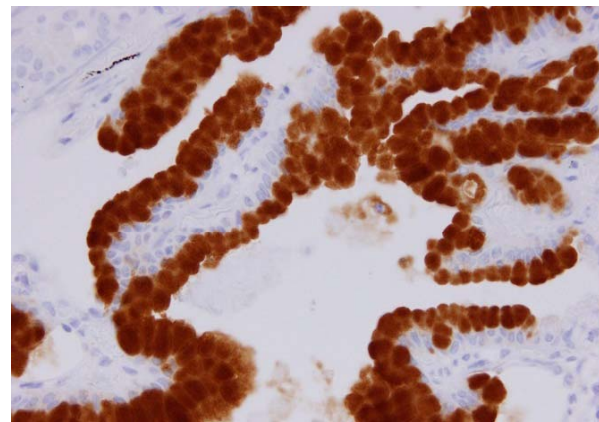
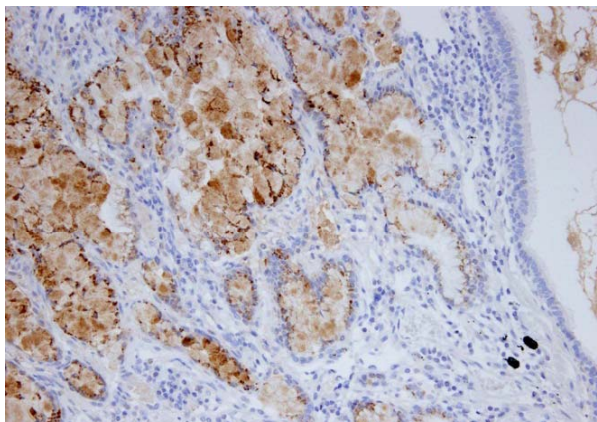
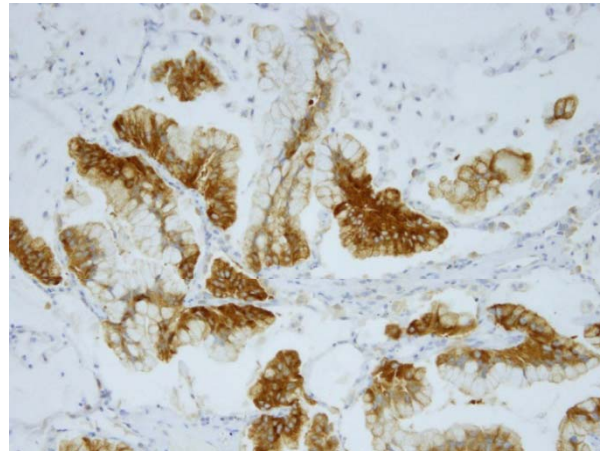
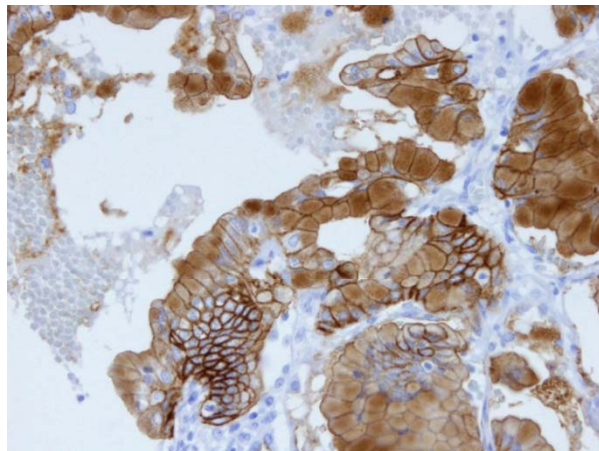
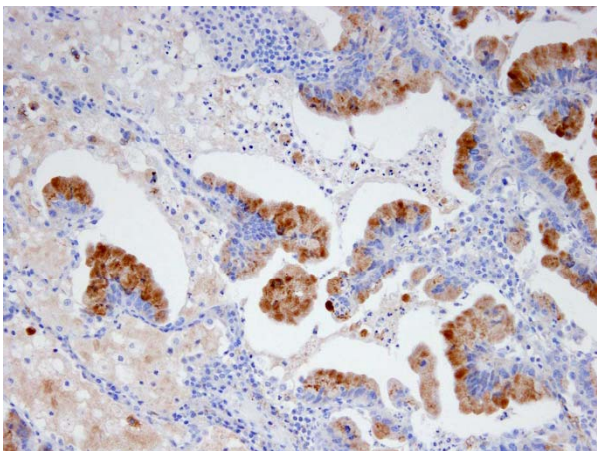
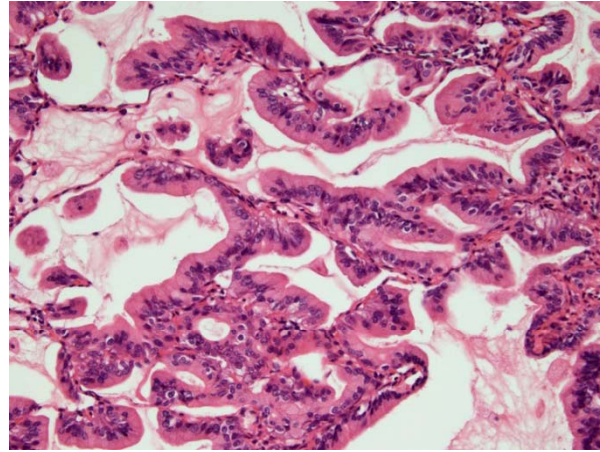


B



C



A**B****C****Figure S5.**

Supplementary Figure Legends

Supplementary Figure S1. Transformation of NIH3T3 cells expressing *CD74-NRG1*, *EZR-ERBB4*, or *TRIM24-BRAF* cDNAs. (A) Expression of gene-fusion products, detected by western-blot analysis, in transiently transfected H1299 cells and virally infected NIH3T3 cells. Antibodies recognizing NRG1 (catalog no: RB-276, Thermo Scientific), ERBB4 (catalog no: 2218-1, Epitomics) and BRAF (catalog no: sc-166, Santa Cruz Biotechnology) polypeptides retained in fusion proteins were used. (B) Photomicrographs of anchorage-independent colony growth of NIH3T3 cells, induced by expression of *CD74-NRG1* (C8;N6), *EZR-ERBB4*, or *TRIM24-BRAF* cDNAs. Scale bar, 100 μm .

Supplementary Figure S2. Electropherogram of Sanger sequencing of cDNAs of novel fusion transcripts. *CD74-NRG1* (two variant forms), *SLC3A2-NRG1*, *EZR-ERBB4*, *TRIM24-BRAF*, and *KIAA1468-RET* fusions. RT-PCR products were sequenced directly.

Supplementary Figure S3. Schematic diagrams of genomic organization for *NRG1*, *ERBB4*, *BRAF*, and *RET* rearrangements. Diagram showing the genomic organization of wild-type and fusion genes, including exons and untranslated regions, for the *CD74-NRG1* (A), *SLC3A2-NRG1* (B), *EZR-ERBB4* (C), *TRIM24-BRAF* (D), and *KIAA1468-RET* (F) fusions. The red and blue boxes show exons, and the arrows show the direction of transcription. The *TRIM24*

and *BRAF* genes are located within 2.2 Mb of each other, in opposite orientations, on chromosome 7; the *TRIM24-BRAF* fusion was deduced to have been generated through paracentric inversion on chromosome 7q33-34 **(B)**. Locations of breakpoints are indicated by green lines.

Supplementary Figure S4. Break-apart fluorescence *in situ* hybridization (FISH) for the *NRG1* fusion. **(A)** Genomic organization of the *NRG1* gene, and the locations of BAC clones used as probes. **(B)** Normal tissue. **(C)** IMA with the *CD74-NRG1* fusion. FISH revealed a separation between the green and orange probes flanking the translocation sites (arrows).

Supplementary Figure S5. Representative histological images obtained from fusion-positive IMAs. Immunostaining was performed using antibodies recognizing polypeptides retained in fusion proteins. **(A)** (Upper) IMA with an *NRG1* rearrangement. The tumor was composed of tall columnar cells with fine eosinophilic cytoplasm and varying amounts of mucin. Nuclear enlargement with fine granular chromatin and dis-alignment of nuclei are visible (original magnification, ×20). (Middle) *NRG1* staining in *CD74-NRG1* fusion-positive IMA. Patchy granular cytoplasmic staining is visible in the adenocarcinoma component (original magnification, ×20). (Lower) *NRG1* staining in fusion-negative IMA (original magnification, ×20). Cytoplasmic granular staining as in fusion-positive tumors was observed in most (>80%) cases. **(B)** (Upper) IMA with an *ERBB4* rearrangement. The tumor was composed of tall columnar cells with basally located small nuclei and mucin in the upper portion

of the cytoplasm (original magnification, ×20). (Middle) ERBB4 staining in *EZR-ERBB4* fusion-positive IMA. Plasma membranous accentuation with cytoplasmic staining is visible (original magnification, ×40). (Lower) ERBB4 staining in fusion-negative IMA (original magnification, ×40). Cytoplasmic staining without membranous accentuation was observed in >50% cases. (C) (Upper) IMA with *BRAF* rearrangement. The tumor was composed of tall columnar cells with condensed eosinophilic mucin. Nuclear enlargement and overlapping of nuclei are visible (original magnification, ×20). (Middle) BRAF staining in a *TRIM24-BRAF* fusion-positive IMA. Diffuse and strong granular cytoplasmic staining is visible in the adenocarcinoma component (original magnification, ×20). (Lower) BRAF staining in fusion-negative IMA (original magnification, ×40). A subset of the cases (<10%) exhibited focal and weak cytoplasmic staining.

Supplementary Table S1. RNA sequencing of 32 IMAs							
No.	Sample ID	Driver oncogene aberration	Library preparation kit	Quantity of RNA used for RNA sequencing (μg)	New generation sequencer	Size of pair-end reads	Novel gene fusions detected by RNA sequencing
1	258T	Pan-negative	mRNA-Seq Kit	2.0	GAllx	50 base PE	
2	AD09-031T	Pan-negative	TruSeq RNA Kit	2.0	HiSeq2000	50 base PE	
3	AD08_220T	KRAS	TruSeq RNA Kit	1.0	GAllx	75 base PE	
4	301T	Pan-negative	TruSeq RNA Kit	1.0	GAllx	75 base PE	CD74-NRG1
5	AD08_127T	Pan-negative	TruSeq RNA Kit	1.0	GAllx	75 base PE	TRIM24-BRAF
6	AD09-398T	Pan-negative	TruSeq RNA Kit	1.0	GAllx	75 base PE	
7	AD12-113T	Pan-negative	TruSeq RNA Kit	1.0	GAllx	75 base PE	
8	AD12-119T	Pan-negative	TruSeq RNA Kit	1.0	GAllx	75 base PE	KIAA1468-RET
9	AD12-121T	KRAS	TruSeq RNA Kit	1.0	GAllx	75 base PE	
10	AD12-127T	KRAS	TruSeq RNA Kit	1.0	GAllx	75 base PE	
11	AD12-129T	Pan-negative	TruSeq RNA Kit	1.0	GAllx	75 base PE	
12	310T	Pan-negative	TruSeq RNA Kit	1.0	GAllx	75 base PE	
13	436T	Pan-negative	TruSeq RNA Kit	1.0	GAllx	75 base PE	EZR-ERBB4
14	AD09-231T	KRAS	TruSeq RNA Kit	1.0	GAllx	75 base PE	
15	AD09-303T	Pan-negative	TruSeq RNA Kit	1.0	GAllx	75 base PE	
16	AD09-317T	KRAS	TruSeq RNA Kit	1.0	GAllx	75 base PE	
17	AD12-108T	Pan-negative	TruSeq RNA Kit	1.0	GAllx	75 base PE	CD74-NRG1
18	AD12-111T	Pan-negative	TruSeq RNA Kit	1.0	GAllx	75 base PE	
19	AD12-112T	Pan-negative	TruSeq RNA Kit	1.0	GAllx	75 base PE	
20	AD12-114T	Pan-negative	TruSeq RNA Kit	1.0	GAllx	75 base PE	
21	AD12-120T	Pan-negative	TruSeq RNA Kit	1.0	GAllx	75 base PE	
22	AD12-126T	Pan-negative	TruSeq RNA Kit	1.0	GAllx	75 base PE	
23	AD13-121T	Pan-negative	TruSeq RNA Kit	1.0	GAllx	75 base PE	
24	AD13-199T	Pan-negative	TruSeq RNA Kit	1.0	GAllx	75 base PE	CD74-NRG1
25	AD13-223T	Pan-negative	TruSeq RNA Kit	1.0	GAllx	75 base PE	CD74-NRG1
26	AD13-227T	Pan-negative	TruSeq RNA Kit	1.0	GAllx	75 base PE	
27	AD13-257T	Pan-negative	TruSeq RNA Kit	1.0	GAllx	75 base PE	
28	AD13-362T	Pan-negative	TruSeq RNA Kit	1.0	GAllx	75 base PE	
29	AD13-364T	Pan-negative	TruSeq RNA Kit	1.0	GAllx	75 base PE	
30	AD13-373T	Pan-negative	TruSeq RNA Kit	1.0	GAllx	75 base PE	
31	AD13-377T	Pan-negative	TruSeq RNA Kit	1.0	GAllx	75 base PE	
32	AD13-379T	Pan-negative	TruSeq RNA Kit	1.0	GAllx	75 base PE	SLC3A2-NRG1

Supplementary Table S2. PCR Primers used for RT-PCR screening

Gene fusion	Forward primer	Reverse primer	RT-PCR product size (bp)
<i>CD74-NRG1</i>	CGGAGAACCTGAGACACCTT	ACTCCCCTCCATTACACAG	285 and 222 for (C8; N6) and (C6; N6), respectively
<i>SLC3A2-NRG1</i>	CAGAAGGATGATGTCGCTCA	ACTCCCCTCCATTACACAG	184
<i>EZR-ERBB4</i>	AAGGAGGAGCTGGAGAGACA	CACCTGAGCCAAGGACTTTT	250
<i>TRIM24-BRAF</i>	TGTCGAGACTGTCAGTTGTTAGAA	GCCCAAATTGATTTTCGATGA	250
<i>KIAA1468-RET</i>	TGTCTCCTGCATTCCATCAA	TCCAAATTCGCCTTCTCCTA	239

1 **Supplementary Table Legends**

2

3 **Supplementary Table 1. RNA sequencing of 32 IMAs**

4

5 **Supplementary Table 2. PCR primers used for RT-PCR screening**

6

Supplementary materials and methods

Samples

IMA patients constituted approximately 2% of all LADC cases who were treated surgically at the National Cancer Center Hospital, Tokyo, Japan, from 1998 to 2013. The resected tissues had been fixed in 10% formalin and embedded in paraffin. Serial 4 μ m sections were stained with hematoxylin and eosin using the Alcian blue–periodic acid Schiff method to visualize cytoplasmic mucin production. Total RNA was extracted from grossly dissected, snap-frozen tissue samples using TRIzol (Invitrogen, Carlsbad, CA, USA), and its quality was examined using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). All samples had RNA Integrity Numbers (RINs) > 6.0. Genomic DNA was also extracted from the tissue samples using the QIAamp DNA Mini kit (Qiagen, Valencia, CA, USA). Hotspot mutations in the *EGFR*, *KRAS*, *BRAF*, and *HER2* genes were examined by the high-resolution melting (HRM) method, and the *EML4*- or *KIF5B*-*ALK*, *KIF5B*- or *CCDC6*-*RET*, and *CD74*-, *EZR*-, or *SLC34A2*-*ROS1* fusions were examined by RT-PCR. Detailed methods were described previously (1-3).

RT-PCR and Sanger sequencing

Total RNA (500 ng) was reverse-transcribed into cDNA using Superscript III Reverse Transcriptase (Invitrogen). cDNA (corresponding to 10 ng total RNA) or 10 ng genomic DNA was subjected to PCR amplification using KAPA Taq DNA Polymerase (KAPA Biosystems, Woburn, MA, USA). The reactions were

carried out in a thermal cycler under the following conditions: 40 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 2 min, with a final extension at 72°C for 10 min. The gene encoding glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was amplified to estimate the efficiency of cDNA synthesis. PCR products were directly sequenced in both directions on an ABI 3130xl DNA Sequencer (Applied Biosystems, Foster City, CA, USA) using the BigDye Terminator kit. The PCR primers used in this study are shown in **Supplementary Table 2.**

Cell lines and reagents

NIH3T3 cells were provided by Dr. T. Yamamoto of Okinawa Institute of Science and Technology, Okinawa, Japan. NCI-H1299 cells were provided by Dr. J. D. Minna of UT Southwestern Medical Center. EFM19 and 293FT cells were obtained from DSMZ (Braunschweig, Germany) and Invitrogen, respectively. H1299 and EFM-19 were cultured in RPMI medium with 10% FBS, and NIH3T3 and 293FT were cultured in DMEM medium with 10% FBS.

Lapatinib, afatinib, and sorafenib were purchased from Selleck (Houston, TX, USA). U0126 was from Calbiochem (San Diego, CA, USA).

Primary antibodies against ERBB4 (catalog no. 2218-1) and ERBB2 (catalog no. 2064-1) were purchased from Epitomics (Burlingame, CA, USA). Antibodies against BRAF (catalog no. sc-166) and ERBB3 (catalog no. sc-285) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). An antibody against the NRG1 EGF-like domain (4) (catalog no. RB-276) was purchased from Thermo Scientific (Fremont, CA, USA). Antibodies against phospho-ERBB4

pTyr1284 (catalog no. 4757), phospho-ERBB3 pTyr1289 (catalog no. 4791), phospho-ERBB2 pTyr1248 (catalog no. 2247), AKT (catalog no. 4691), phospho-AKT pSer473 (catalog no. 4060), total ERK1/2 (catalog no. 4695), phospho-ERK1/2 pThr202/Tyr204 (catalog no. 4370), and beta-actin (catalog no. 3700) were purchased from Cell Signaling Technology (Danvers, MA, USA).

Immunohistochemistry

Immunohistochemistry was performed on tissue microarray sections. Sections 4µm thick were deparaffinized, and heat-induced epitope retrieval was performed using targeted retrieval solution 9 (Dako, Carpinteria, CA, USA) for BRAF, and NRG and citrate buffer for ERBB4. The slides were treated with 3% hydrogen peroxide for 20 min to block endogenous peroxidase activity, followed by washing in deionized water for 2–3 min. The slides were then incubated with primary antibodies against BRAF (1:800, polyclonal, Sigma, St. Louis, Mo, USA), NRG1 (1:500, polyclonal, Thermo Scientific), or ERBB4 (1:100, clone E200; Abcam, Cambridge, UK) for 1 hr at room temperature. Immunoreactions were detected using the EnVision-FLEX and LINKER (Dako) systems. The reactions were visualized with 3,3'-diaminobenzidine, followed by counterstaining with hematoxylin. Cytoplasmic staining of >10% of tumor cells was considered positive for BRAF and NRG1, and membrane staining was considered positive for ERBB4.

Fluorescence *in situ* hybridization

To identify *NRG1* rearrangements, fluorescence *in situ* hybridization (FISH) was

performed on formalin-fixed, paraffin-embedded tumors using a break-apart probe for *NRG1* (Chromosome Science Labo, Sapporo, Japan; RP11-1002K11 + RP11-35D16, labeled with Spectrum Orange, as a 3' centromeric probe, and RP11-23A12 + RP11-715M18, labeled with Spectrum Green, as a 5' telomeric probe).

Construction of lentiviral vectors for expression of CD74-NRG1, EZR-ERBB4 and TRIM24-BRAF fusion proteins

Full-length *CD74-NRG1*, *EZR-ERBB4*, and *TRIM24-BRAF* cDNAs were obtained by PCR amplification of cDNAs from each index tumor sample using KOD-PLUS Taq polymerase (Toyobo, Osaka, Japan). The products were digested with restriction endonucleases and ligated into pLenti-6/V5-DEST plasmids (Invitrogen). The integrity of each inserted cDNA was verified by Sanger sequencing. Lentiviruses expressing *CD74-NRG1*, *EZR-ERBB4*, or *TRIM24-BRAF* were produced by transfecting each expression plasmid into 293FT cells along with ViraPower packaging mix (Invitrogen) using the Lipofectamine 2000 reagent (Invitrogen).

For transient expression, empty plasmids or plasmids expressing *CD74-NRG1*, *TRIM24-BRAF*, or *EZR-ERBB4* cDNA were transfected into NCI-H1299 lung cancer cells at 80% confluence using the Lipofectamine 2000 reagent. After a 24 h incubation in supplemented RPMI medium, cells were used for assays.

For stable expression, NIH3T3 fibroblasts at 60–70% confluence were infected with empty, *CD74-NRG1*-, *EZR-ERBB4*-, or *TRIM24-BRAF*-expressing

lentiviruses, and then treated with blasticidin (4 µg/ml) for 2 weeks. Mass-cultured blasticidin-resistant cells were used for assays.

HER2:HER3 signaling activation by *CD74-NRG1*

To determine whether cells expressing *CD74-NRG1* cDNA secreted NRG1 ligands that activate HER2:HER3 intracellular signaling, EFM-19 breast cancer cells, which express both the ERBB2/HER2 and ERBB3/HER3 proteins, were used as reporter cells, as previously described (4). NCI-H1299 cells transiently transfected with *CD74-NRG1* expression plasmid or empty (control) plasmid were washed twice with PBS and serum-starved overnight by incubation in serum-free medium. Media were harvested and centrifuged for 5 min at 4°C to remove cell debris. Sub-confluent EFM-19 cells were incubated for 30 min with these conditioned media, supplemented with DMSO (vehicle control) or HER-TKIs. Whole-cell lysates were then subjected to SDS-PAGE and immunoblotting.

Constitutive activation of ERBB4 and BRAF kinases and its inhibition by tyrosine kinase inhibitors

NIH3T3 cells stably transfected with plasmids expressing *EZR-ERBB4* or *TRIM24-BRAF* cDNAs, or with empty plasmids, were maintained in serum-free medium overnight, and then treated with DMSO (Sigma) or the indicated inhibitor (dissolved in DMSO) for 2 h. Whole-cell lysates were subjected to immunoblot analysis.

Immunoblotting

Cells were lysed in RIPA buffer containing Complete Protease and PhosSTOP Phosphatase Inhibitor Cocktail (Roche, Mannheim, Germany). Proteins were subjected to SDS-PAGE, followed by immunoblotting onto polyvinylidene difluoride membranes. The membranes were blocked for 1 h with TBS containing 0.1% Tween 20 and 1.0% BSA, and then probed with primary antibodies. After washing with TBS containing 0.1% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies, and then visualized with an enhanced chemiluminescence reagent (Perkin Elmer, Waltham, MA, USA). Intensities of signals were calculated using an LAS3000 imaging system (Quansys Biosciences, West Logan, UT, USA).

Soft-agar assay

NIH3T3 cells infected with empty, *CD74-NRG1*, *EZR-ERBB4*, or *TRIM24-BRAF* lentiviruses were seeded in triplicate at 4,000 cells per well in 24-well plates in a top layer of 0.3% SeaPlaque agarose (Lonza, Rockland, ME, USA) on a base layer of 0.6% agarose. Media containing DMSO or tyrosine kinase inhibitors were added to the top agar, as well as on top of the 0.3% agarose layer. Cover media were replaced twice a week. Colonies larger than 100 μm in diameter were counted 14 days later.

Tumorigenicity assay in nude mice

Stable NIH3T3 cells (5×10^6) harboring empty vector or vectors expressing

EZR-ERBB4 or TRIM24-BRAF fusion protein were resuspended in PBS with 50% Matrigel (BD Biosciences, Bedford, MA, USA). Cells were injected subcutaneously into the right flank of 6-week-old female *nu/nu* mice. Tumor size was measured twice weekly until tumor size reached approximately 2 cm × 2 cm. Photographs were taken on day 21. All studies involving mice were approved by the Ethical Committee on Animal Experiments at the National Cancer Center.

References

1. Kohno T, Ichikawa H, Totoki Y, Yasuda K, Hiramoto M, Nanno T, et al. KIF5B-RET fusions in lung adenocarcinoma. *Nat Med*. 2012;18:375-7.
2. Yoshida A, Kohno T, Tsuta K, Wakai S, Arai Y, Shimada Y, et al. ROS1-rearranged lung cancer: a clinicopathologic and molecular study of 15 surgical cases. *Am J Surg Pathol*. 2013;37:554-62.
3. Kinno T, Tsuta K, Shiraishi K, Mizukami T, Suzuki M, Yoshida A, et al. Clinicopathological features of nonsmall cell lung carcinomas with BRAF mutations. *Ann Oncol*. 2014;25:138-42.
4. Wilson TR, Lee DY, Berry L, Shames DS, Settleman J. Neuregulin-1-mediated autocrine signaling underlies sensitivity to HER2 kinase inhibitors in a subset of human cancers. *Cancer Cell*. 2011;20:158-72.