factors (7 of 109), both good candidates for molecules involved in the specification of guard cells (fig. S3B). In addition to genes required for guard cell specification, we predicted that transcripts for genes required for guard cell function would appear in this transcriptional cluster. The cell walls of differentiated guard cells are extensively reinforced, and correspondingly, several transcripts (11%, or 12 of 109) were annotated as cell wall modification proteins (fig. S3B).

Sequence-indexed transferred (T-DNA) insertion lines are currently available for ~70% of the transcripts identified in the microarray experiments (*18*). To validate this genomic approach to identifying new stomatal development genes, we characterized the phenotype of 160 T-DNA insertions that represent 82 of 220 differentially expressed genes. Ten loci exhibited stomatal or epidermal phenotypes in at least two independent insertion lines (*9*). A marked phenotype was observed in lines homozygous for insertions in a putative transcription factor, At3g24140 (*FAMA*) (Fig. 4, C and D). *FAMA* exhibited a transcriptional profile similar to that of *SDD1* and *HIC1* (Fig. 4B). Existing microarray data revealed that *FAMA* expression is consistent with a role in stomatal pattern: highest in leaves (Affymetrix values 315 ± 13), lower in flowers (212 ± 17), and absent in roots (44 ± 33) (*15, 19*). The cotyledon epidermis of *fama1-1* (SALK_100073) (*18*) had no recognizable guard cells. Instead, chains of small cells that rarely make obvious stomatal pores but that, like guard cells (and unlike pavement cells), often contain mature chloroplasts were intercalated among pavement cells (Fig. 4, E and F). These clusters of incompletely differentiated cells are reminiscent of, but larger than, the clusters of mature guard cells found in *flp* mutants (*10*). *FLP* has been proposed to limit GMC division competence cell-autonomously (*1*). Based on the up-regulation of *FAMA* in plants that overproduce stomata, it is likely that *FAMA* plays a similar cell-autonomous role in regulating GMC behavior.

We have shown that *YDA* regulates a fundamental cell fate decision in the *Arabidopsis* epidermis. *YDA* activity must be down-regulated to allow cells to enter the stomatal lineage, and its normal activity is required to maintain the balance of proliferation versus differentiation (into GMCs) in the meristemoids (Fig. 4G). In animal cell fate decisions, MAPK signaling cascades often act as molecular switches by converting small changes at the cell periphery into multiple downstream responses (*20*). Stomatal cell fate may also require such an amplification mechanism because only some protodermal cells are initially chosen to enter the stomatal lineage and their recruitment is not predicted by any obvious character of those cells. The continued requirement for *YDA* in meristemoids may be tied to a universally conserved role of MAPK signaling in controlling cell cycle progression (*21*) or may

reflect the utility of MAPK signaling for responses to changing environmental stimuli, as best characterized in the *Saccharomyces cerevisiae* osmotic response (*22*) and in *Arabidopsis* hormone (*23, 24*) and pathogen (*25*) responses.

Plants respond to global climate factors such as CO_2 and water and light availability by adjusting their stomatal density, stomatal index, and stomatal distribution (*26*). These developmental responses have complex effects on plant fitness and water use efficiency (*27*). We have shown the utility of the *YDA* system to identify new stomatal cell fate regulators. Additionally, the ability to use *YDA* to manipulate stomatal density in a model plant species will facilitate an analysis of the mechanisms by which stomatal density is controlled and the physiological relevance of the large variation in density found in nature.

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EGFR Mutations in Lung Cancer: Correlation with Clinical Response to Gefitinib Therapy

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Receptor tyrosine kinase genes were sequenced in non–small cell lung cancer (NSCLC) and matched normal tissue. Somatic mutations of the epidermal growth factor receptor gene *EGFR* were found in 15 of 58 unselected tumors from Japan and 1 of 61 from the United States. Treatment with the EGFR kinase inhibitor gefitinib (Iressa) causes tumor regression in some patients with NSCLC, more frequently in Japan. *EGFR* mutations were found in additional lung cancer samples from U.S. patients who responded to gefitinib therapy and in a lung adenocarcinoma cell line that was hypersensitive to growth inhibition by gefitinib, but not in gefitinib-insensitive tumors or cell lines. These results suggest that *EGFR* mutations may predict sensitivity to gefitinib.

Protein kinase activation by somatic mutation or chromosomal alteration is a common mechanism of tumorigenesis (*1*). Inhibition of activated protein kinases through the use of targeted small molecule drugs or antibody-based strategies has emerged as

an effective approach to cancer therapy (*2–4*). Recently, systematic analysis of kinase genes has identified mutations of the protein serine-threonine kinase gene *BRAF* in melanoma and other human cancers (*5*) and of multiple tyrosine kinase genes and the phosphatidylinositol 3-kinase p110α catalytic subunit gene *PIK3CA* in human colorectal carcinoma (*6, 7*).

Lung carcinoma is the leading cause of cancer deaths in the United States and worldwide for both men and women (*8*). Chemotherapy for non–small cell lung carcinoma (NSCLC), which accounts for approximately 85% of lung cancer cases, remains marginally effective (*9*).

Recently, the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, gefitinib (Iressa), was approved in Japan and the United States for the treatment of NSCLC. The original rationale for its use was the observation that EGFR is more abundantly expressed in lung carcinoma tissue than in adjacent normal lung (*10*). However, EGFR expression as detected by immunohistochemistry is not an effective predictor of response to gefitinib (*11*).

Clinical trials have revealed significant variability in the response to gefitinib, with higher responses seen in Japanese patients than in a predominantly European-derived population (27.5% versus 10.4%, in a multi-institutional phase II trial) (*12*). In the United States, partial clinical responses to gefitinib have been observed most frequently in women, in nonsmokers, and in patients with adenocarcinomas (*13–15*).

To determine whether mutation of receptor tyrosine kinases plays a causal role in NSCLC, we searched for somatic genetic alterations in a set of 119 primary NSCLC tumors, consisting of 58 samples from Nagoya City University Hospital in Japan and 61 from the Brigham and Women's Hospital in Boston, Massachusetts. The tumors included 70 lung adenocarcinomas and 49 other NSCLC tumors from 74 male and 45 female patients, none of whom had documented treatment with gefitinib.

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As an initial screen, we amplified and sequenced the exons encoding the activation loops of 47 of the 58 human receptor tyrosine kinase genes (*16*) (table S1) from genomic DNA from a subset of 58 NSCLC samples that included 41 lung adenocarcinomas. Three of the tumors, all lung adenocarcinomas, showed heterozygous missense mutations in *EGFR* not present in the DNA from normal lung tissue from the same patients (table S2; S0361, S0388, S0389). No mutations were detected in amplicons from other receptor tyrosine kinase genes. All three tumors had the same *EGFR* mutation, predicted to change leucine-858 to arginine (Fig. 1A; CTG→CGG; L858R).

We next examined exons 2 through 25 of *EGFR* in the complete collection of 119 NSCLC tumors. Exon sequencing of genomic DNA revealed missense and deletion mutations of *EGFR* in a total of 16 tumors, all within exons 18 through 21 of the kinase domain. All sequence alterations in this group were heterozygous in the tumor DNA; in each case, paired normal lung tissue from the same patient showed wild-type sequence, confirming that the mutations are somatic in origin. The distribution of nucleotide and protein sequence alterations, and the patient characteristics associated with these abnormalities, are summarized in table S2.

Substitution mutations G719S and L858R were detected in two and three tumors, respectively. These mutations are located in the
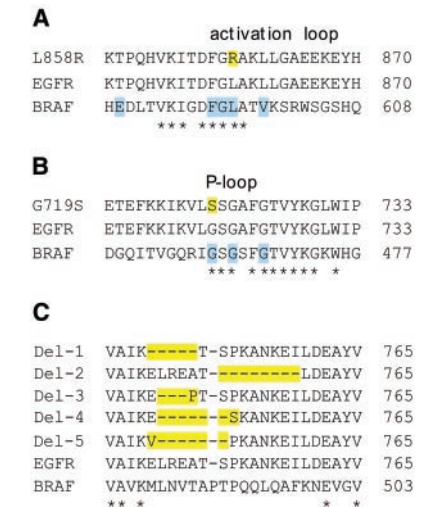

Fig. 1. Sequence alignment of selected regions within the EGFR and B-Raf kinase domains. Depiction of each type of EGFR mutation in human NSCLC. EGFR (gb:X00588) mutations in NSCLC tumors are highlighted in yellow. B-Raf (gb:M95712) mutations in multiple tumor types (*5*) are highlighted in blue. Asterisks denote residues conserved between EGFR and B-Raf. (**A**) L858R mutations in activation loop. (**B**) G719S mutant in P-loop. (**C**) Deletion mutants in EGFR exon 19.

GXGXXG motif of the nucleotide triphosphate binding domain or P-loop and adjacent to the highly conserved DFG motif in the activation loop (*17*), respectively. The mutated residues are nearly invariant in all protein kinases, and the analogous residues (G463 and L596) in the B-Raf protein serine-threonine kinase are somatically mutated in colorectal, ovarian, and lung carcinomas (*5, 18*) (Fig. 1, A and B).

We also detected multiple deletion mutations clustered in the region spanning codons 746 to 759 within the kinase domain of EGFR. Ten tumors carried one of two overlapping 15-nucleotide deletions eliminating *EGFR* codons 746 to 750, starting at nucleotide 2235 or 2236 (Del-1) (Fig. 1C and table S2). *EGFR* DNA from another tumor displayed a heterozygous 24-nucleotide gap leading to the deletion of codons 752 to 759 (Del-2) (Fig. 1C). Representative chromatograms are shown in fig. S1.

The positions of the substitution mutations and the Del-1 deletion in the three-dimensional structure of the active form of the EGFR kinase domain (*19*) are shown in Fig. 2. Note that the sequence alterations cluster around the active site of the kinase and that the substitution mutations lie in the activation loop and glycine-rich P-loop, structural elements known to be important for autoregulation in many protein kinases (*17*).

The *EGFR* mutations show a striking correlation with patient characteristics. Mutations were more frequent in adenocarcinomas (15/70 or 21%) than in other NSCLCs (1/49 or 2%), more frequent in women (9/45 or 20%) than in men (7/74 or 9%), and more frequent in the patients from Japan (15/58 or 26%, and 14/41 adenocar-
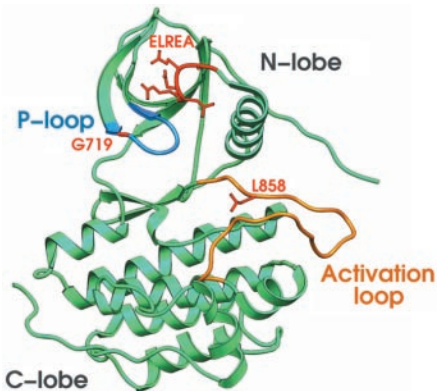
Fig. 2. Positions of missense mutations G719S and L858R and the Del-1 deletion in the three-dimensional structure of the EGFR kinase domain. The activation loop is shown in yellow, the P-loop is in blue, and the C-lobe and N-lobe are as indicated. The residues targeted by mutation or deletion are highlighted in red. The Del-1 mutation targets the residues ELREA in codons 746 to 750.

cinomas or 32%) than in those from the United States (1/61 or 2%, and 1/29 adenocarcinomas or 3%). The highest fraction of *EGFR* mutations was observed in Japanese women with adenocarcinoma (8/14 or 57%). Notably, the patient characteristics that correlate with the presence of *EGFR* mutations are those that correlate with clinical response to gefitinib treatment.

To investigate whether *EGFR* mutations might be a determinant of gefitinib sensitivity, pretreatment NSCLC samples were obtained from 5 patients who responded and 4 patients who progressed during treatment with gefitinib out of more than 125 patients treated at the Dana-Farber Cancer Institute either on an expanded access program or after regulatory approval of gefitinib (*13*). Four of the patients had partial radiographic responses (≥50% tumor regression in a computed tomography scan after 2 months of treatment), whereas the fifth patient experienced dramatic symptomatic improvement in less than 2 months. All of the patients were from the United States and were Caucasian.

While sequencing of the kinase domain (exons 18 through 24) revealed no mutations in tumors from the four patients who progressed on gefitinib, all five tumors from gefitinib-responsive patients harbored *EGFR* kinase domain mutations. The chi-square test revealed the difference in *EGFR* mutation frequency between gefitinib responders (5/5) and nonresponders (0/4) to be statistically significant with $P = 0.0027$, whereas the difference between the gefitinib responders and unselected U.S. NSCLC patients (5/5 versus 1/61) was also significant with $P < 10^{-12}$ (*20*). The *EGFR* L858R mutation, previously observed in the unselected tumors, was identified in one gefitinib-sensitive lung adenocarcino-

ma (Fig. 1A and table S3, IR3T). Three gefitinib-sensitive tumors contained heterozygous in-frame deletions (Fig. 1C and table S3, Del-3 in two cases and Del-4 in one), and one contained a homozygous in-frame deletion (Fig. 1C and table S3, Del-5). Each of these deletions was found within codons 746 to 753 of *EGFR*, where deletions were also found in unselected tumors. Each of these three deletions is also associated with an amino acid substitution (table S3). In all four samples where matched normal tissue was available, these mutations were confirmed as somatic.

To determine whether mutations in *EGFR* confer gefitinib sensitivity in vitro, the mutation status and response to gefitinib were determined in four lung adenocarcinoma and bronchioloalveolar carcinoma cell lines. The H3255 cell line was originally derived from a malignant pleural effusion from a Caucasian female nonsmoker with lung adenocarcinoma (*21*). This cell line was 50 times as sensitive to gefitinib as the other lines, with an IC50 of 40 nM for cell survival in a 72-hour assay (Fig. 3A).

Treatment with 100 nM gefitinib completely inhibited EGFR autophosphorylation in H3255 (Fig. 3B). Such treatment also inhibited the phosphorylation of known downstream targets of EGFR such as the extracellular signal-regulated kinase 1/2 (ERK1/2) and the v-akt murine thymoma viral oncogene homolog (AKT kinase) (Fig. 3B), a correlation that has been noted by others (*22*). In contrast, the other three cell lines showed comparable levels of inhibition of target protein phosphorylation only when gefitinib was present at concentrations roughly 100 times as high (Fig. 3B).

The sequence analysis of *EGFR* cDNA in these four cell lines showed the L858R

mutations in H3255 (table S3), whereas the other three cell lines did not contain *EGFR* mutations. We also confirmed the presence of the L858R mutation in the primary tumor from which H3255 was derived (table S3, IRG), although no matched normal tissue was available. The results suggest that L858R mutant EGFR is particularly sensitive to inhibition by gefitinib compared with the wild-type enzyme and that this likely accounts for the extraordinary drug sensitivity of the H3255 cell line.

The identification of *EGFR* mutations in a subset of human lung carcinomas and the association between *EGFR* mutation and gefitinib sensitivity extend the emerging paradigm whereby genetic alterations in specific kinases, and not simply kinase expression, render tumors sensitive to selective inhibitors as is the case for imatinib treatment of *c-kit* mutant gastrointestinal stromal tumors (*23*). Thus, although randomized trials of cytotoxic therapy with or without gefitinib revealed no survival benefit for the gefitinib-treated NSCLC patients (*24*, *25*), our current data suggest that gefitinib may be particularly effective for treating lung cancers with somatic *EGFR* mutations and that prospective clinical trials of EGFR inhibition in patients with *EGFR* mutations might reveal increased patient survival. Identification of *EGFR* mutations in other malignancies, perhaps including glioblastomas in which *EGFR* alterations are already known (*26*), may identify other patients who could similarly benefit from treatment with EGFR inhibitors.

Important questions remain to be answered, including whether these alterations result in activated and transforming alleles of *EGFR*, whether receptors harboring such mutations will show differential sensitivity to any of the multiple EGFR small molecule
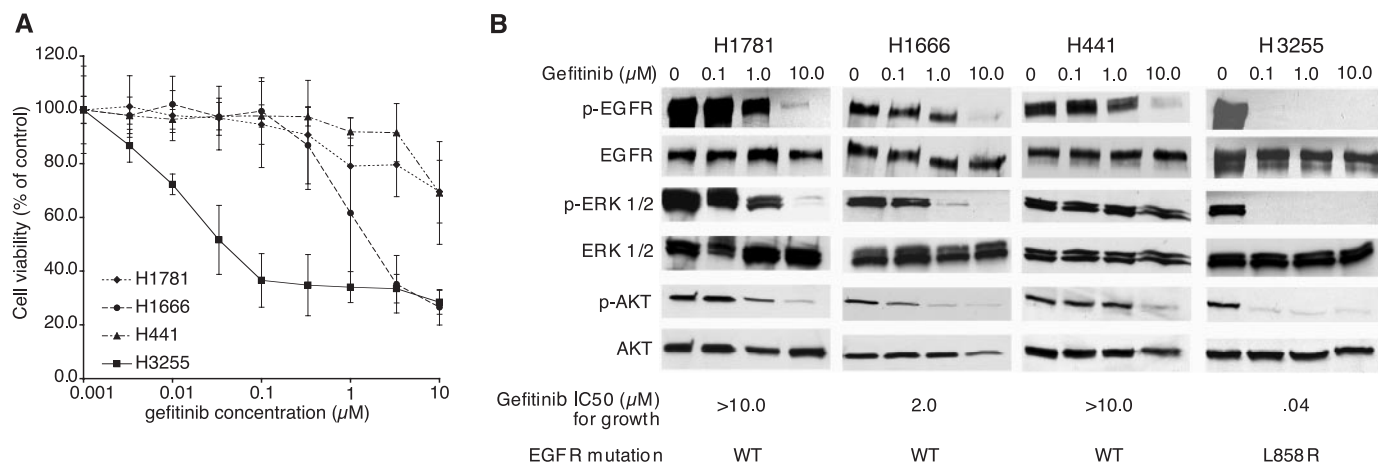
Fig. 3. A lung adenocarcinoma cell line with EGFR receptor mutation is sensitive to growth and signaling inhibition by gefitinib. (**A**) Cells were treated with gefitinib at the indicated concentrations, and viable cells were measured after 72 hours of treatment. Percentage of cell growth is shown relative to untreated controls. H3255 cells have the EGFR L858R mutation, whereas the three remaining cell lines have wild-type EGFR (WT). (**B**) Inhibition of EGFR phosphorylation and of downstream phosphorylation of Akt and Erk1/2. The cell lines were treated with gefitinib for 24 hours. Cell extracts were immunoblotted to detect the indicated protein species. Akt, v-akt murine thymoma viral oncogene homolog; Erk, extracellular signal-responsive kinase.

inhibitors, and whether EGFR receptors harboring such mutations are inhibited by antibodies directed against the extracellular domain. Furthermore, it will be of interest to determine whether resistance to EGFR inhibition emerges through secondary mutation as is the case in imatinib-treated chronic myelogenous leukemia (27). These results should stimulate further in vitro studies regarding these questions.

Finally, the striking differences in the frequency of *EGFR* mutation and response to gefitinib between Japanese and U.S. patients raise general questions regarding variations in the molecular pathogenesis of cancer in different ethnic, cultural, and geographic groups and argue for the benefit of population diversity in cancer clinical trials.

Note added in proof: Similar results are being reported by T. J. Lynch et al. (28).

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20. Note that the frequency of EGFR mutation in the unselected U.S. patients, 1 of 61, appears to be low when compared with the frequency of reported gefitinib response at 10.4%. This difference has a modest statistical significance ($P = 0.025$ by the chi-square test). Thus, this result could still be due to chance, to a fraction of responders who do not have EGFR mutations, or to failure to detect EGFR mutations experimentally in this tumor collection. If the frequency of EGFR mutation in gefitinib-responsive U.S. patients (5/5) is compared with the expected frequency of gefitinib response (10.4%), the chi-square probability is again less than 10^{-12}.
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Regulation of an *ATG7–beclin 1* Program of Autophagic Cell Death by Caspase-8

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Caspases play a central role in apoptosis, a well-studied pathway of programmed cell death. Other programs of death potentially involving necrosis and autophagy may exist, but their relation to apoptosis and mechanisms of regulation remains unclear. We define a new molecular pathway in which activation of the receptor-interacting protein (a serine-threonine kinase) and Jun amino-terminal kinase induced cell death with the morphology of autophagy. Autophagic death required the genes *ATG7* and *beclin 1* and was induced by caspase-8 inhibition. Clinical therapies involving caspase inhibitors may arrest apoptosis but also have the unanticipated effect of promoting autophagic cell death.

Apoptosis is a well-studied pathway of programmed cell death conserved from *Caenorhabditis elegans* to humans (1). Caspases, a family of cystinyl aspartate-requiring proteases, produce the morphological changes associated with apoptotic death (2, 3). Nonapoptotic forms of cell elimination include those with features of necrosis and autophagy (4–7). Necrosis can result when cell metabolism and integrity are compromised by a nonphysiological insult. Recently, evidence has emerged that death receptors and receptor-interacting protein (RIP) can induce caspase-independent cell death that appears necrotic (6, 7). Autophagy promotes a cell survival response to nutritional starvation involving membrane-bound vacuoles that target organelles and proteins to the lysosome for degradation (8, 9). Two pathways functioning in autophagy contain ubiquitin-like genes that are highly conserved from yeast to humans (*ATG* genes). Certain examples of cell death have autophagic features, but a role for *ATG* genes in cell death has not been established (10).

In mouse L929 fibroblastic cells, tumor necrosis factor, oxidants, ceramide, and radiation can induce caspase-independent death (11). However, benzyloxycarbonyl-valyl-alanyl–aspartic acid (O-methyl)–fluoro-methylketone (zVAD), a caspase inhibitor with broad specificity, also directly induced the death of L929 cells. Death began at 12 hours after zVAD treatment and was complete after 40 hours (Fig. 1, A and B). The dead cells appeared to be round and detached, and they had a convoluted plasma membrane permeable to vital dyes; this differed from apoptosis, in which nuclei are condensed and membrane integrity is preserved. Transmission electron microscopy (TEM) revealed intact mitochondria and endoplasmic reticulum, condensed osmophilic cytoplasm, and numerous large cytoplasmic inclusions that were membrane-bound vacuoles characteristic of autophagy (Fig. 1C). A time course revealed that vacuolated cells accumulated before cell death (Fig. 1D). Similar results were obtained in human U937 monocytoid cells (Fig. 1E and fig. S2). The zVAD treatment also induced cell death in mouse RAW 264.7 macrophage cells and primary mouse peritoneal macrophages (figs. S3 and S4).

The association of autophagic vacuoles with cell death has been observed in developing animals, but it has not been clear whether the process serves to rescue or condemn the cell (12). *Drosophila* cells mani-

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