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The discovery of *EGFR* kinase domain mutations in NSCLC patients who responded to tyrosine kinase inhibitors (TKIs) represented the first example of a targeted therapy for lung cancer. The dependence of these human tumors on sustained mutant receptor expression for survival, together with the discovery that ectopic expression of the receptor resulted in transformation, suggested that these mutations are causal events, and as such would be sufficient to induce tumor formation in the lung. To investigate this, and to further our understanding of how deregulated signaling through the mutant receptor could initiate tumor formation, we generated both a conditional and constitutive knock-in allele of one such mutation, L858R, at the endogenous murine *Egfr* locus. Expression of mutant Egfr failed to induce lung tumors in these mice; further analysis of the germline mutant mice revealed significant downregulation of the mutant receptor, and this was predominantly at the post-transcriptional level. These data suggest that normal cells can respond to an oncogenic lesion by upregulating negative feedback pathways to counteract the induction of aberrant signaling, and disabling these feedback mechanisms may be an essential component of the progression of *EGFR* mutant tumors.

A multitude of positive and negative feedback loops converge on signaling pathways to ensure the appropriate output in response to a given stimulus. The role of oncogenes is typically thought of in terms of increasing the output of a signaling pathway, and while the contribution of the associated negative feedback loops is no doubt important, they have been afforded little attention. Recent studies have highlighted the existence of negative feedback mechanisms in established tumors and the integral role they play in shaping the signaling network, with a corresponding appreciation for how such feedback loops can profoundly influence therapeutic response. The capacity of negative regulators to modulate the oncogenic potential of a mutant protein in the context of tumor initiation has rarely been examined. Using a doxycycline-inducible system we recapitulate the aforementioned downregulation of mutant Egfr, initially observed in both tissues and MEFs derived from $Egfr^{L858R}$ mutant mice, in an ectopic cell culture expression system. We establish a role for ERK pathway signaling, and specifically DUSP6, in receptor degradation, and solidify the role of the E3 ligase, CULLIN5, in the downregulation of the mutant receptor. The existence of these negative feedback loops may explain the observation that mutation of EGFR is often coincident with gene amplification in NSCLC, and suggest that such amplification may primarily serve as a means to counteract the downregulation.

The amplification of oncogenes is a recurring feature of many human tumors, but the contribution of gene amplification to particular stages of tumor development, or the molecular requirements for amplification to occur are unknown. EGFR is mutated and coincidentally amplified in NSCLC, but the relative contribution of mutation and amplification, both to tumor phenotype and therapeutic sensitivity, is not clear. The inability to model amplification in the mouse has contributed significantly to our limited mechanistic understanding of how gene amplification occurs in tumors. Using a yeast endonuclease, I-Sce1, and an allele that contains

target sites for this enzyme engineered telomeric to mutant *Egfr* on chromosome 11, we attempted to initiate breakage-fusion-bridge (BFB) cycles in the lung, as these are thought to be a precursor to gene amplification. Our inability to elicit tumor formation using this strategy highlights the limitations in our understanding of how amplicons form in human tumors or the particular context required. While it would provide tremendous insight into mutant EGFR tumor development, a model of targeted gene amplification has thus far eluded us, and remains one of the significant challenges facing the mouse modeling community.

Thesis Supervisor: Tyler Jacks, PhD
Title: Professor of Biology

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Chapter 1: Introduction

Epidermal growth factor receptor (EGFR)

The discovery of growth factors

Cellular decisions, from unicellular through metazoan lineages, initiate primarily through the detection of extracellular signals, followed by the interpretation of such signals, which results in a coordinated and appropriate output or response, ranging from growth or differentiation, to migration or apoptosis. Cell surface receptors, including receptor tyrosine kinases (RTKs), represent an essential component of this fundamental cellular decision making process. The study of growth factor receptors has its origins in the seminal work of Rita Levi-Montalcini in the 1950s, which led to the identification of the first metazoan growth factor, nerve growth factor (NGF). It was originally isolated from mouse sarcomas transplanted into chick embryos, and later purified from mouse salivary glands and snake venom (Cohen and Levi-Montalcini, 1957). Stanley Cohen, while working with Levi-Montalcini on NGF purification from salivary glands, found precocious eye opening and tooth eruption when newborn mice were injected with unpurified NGF from salivary glands (Cohen, 1962). He went on to identify the responsible factor from mouse salivary glands, and coined the term epidermal growth factor (EGF) as a result of its effect on epidermal tissues (Cohen, 1965). Cohen used ^{125}I-EGF to confirm that it bound to a surface protein on fibroblasts (Carpenter et al., 1975), and along with the subsequent identification of its cognate receptor, epidermal growth factor receptor (EGFR), launched the ensuing thirty years of investigation into the ERBB family.

Receptor tyrosine kinases

There are approximately 500 kinases encoded in the human genome, the vast majority of which are serine/threonine kinases. Studies on polyoma middle T antigen and the SRC kinase identifying tyrosine as a third amino acid that could be phosphorylated (Eckhart et al., 1979; Hunter and Sefton, 1980), led to the designation of a new subgroup, the tyrosine kinases (TKs). In humans and mouse there are ninety tyrosine kinases, of which fifty-eight are receptor

tyrosine kinases, composing 20 subfamilies. Tyrosine kinases have been shown to play critical roles in many aspects of normal development and physiology, and although they represent only 0.3% of the genome they are mutated, or otherwise deregulated, in a disproportionate number of human tumors (Blume-Jensen and Hunter, 2001). Indeed, the first viral oncogene cloned, v-SRC, was shown to be a kinase (Collett and Erikson, 1978; Levinson et al., 1978), thus establishing an association between aberrant phosphorylation and tumor formation, in this case avian sarcomas. The deregulation of tyrosine kinase activity has since been found to contribute to a range of disease states including cancer, marking them as attractive candidates for therapeutic targeting. The phosphorylation of tyrosine residues on receptors upon ligand binding was a pivotal discovery; it identified the information transfer code a cell uses to register the detection of extracellular signals and relay that signal internally so that it can be transmitted into an appropriate response (Hunter and Cooper, 1981). Tyrosine phosphorylation acts as a regulatory switch, reversibly flipping cells between non-responsive and responsive states; therefore, phosphorylation of tyrosine needs to be carefully regulated to avoid inappropriate signaling.

Although the need to sense and respond to the state of the local environment is a universal feature of cells, tyrosine kinases are mostly absent from bacteria, *Saccharomyces cerevisiae* and *Arabidopsis thaliana*, and apart from *Choanoflagellates, Chlamydomonas reinhardtii, Phytophthora infestans,* and *Entamoeba histolytica*, are restricted to metazoan lineages (Shiu and Li, 2004). A key distinction between the invertebrate and vertebrate lineages is the disproportionate vertebrate expansion of the RTK family compared with the shift in the overall number of either kinases or cellular tyrosine kinases (Amit et al., 2007b). This two-to four-fold increase in RTKs, proposed to be of functional significance in vertebrate evolution, is thought to have resulted from either two rounds of whole genome duplication or multiple partial chromosomal duplications. It has recently been demonstrated that the number of tyrosine kinases encoded by a genome positively correlates with the number of cell types in an

organism, while the number of tyrosines specified in the genome negatively correlates with both organismal complexity and the number of tyrosine kinases encoded (Tan et al., 2009). This may be a reflection of the potent nature of phospho-tyrosine as a signaling entity, and may also represent an adaptation to a new signaling modality, with the target amino acid being eliminated to avoid aberrant signaling that could have detrimental consequences. The correlation between tyrosine kinases and organismal complexity, along with the high degree of conservation of kinases between species, substantiates the role of such kinases in the coordination of information processing and decision-making in multicellular organisms.

Initial connections between the ERBB family and cancer

EGFR, the founding member of the ERBB family, is the prototypic receptor tyrosine kinase. *EGFR* was originally cloned from a human epidermoid carcinoma cell line, A431 (Ullrich et al., 1984), and found to have homology to a previously discovered viral oncogene, *v-erbB*, shown to cause erythroblastosis and sarcomas in chickens (Downward et al., 1984; Yamamoto et al., 1983). A comparison with EGFR found v-erbB to be missing most of the extracellular domain along with a 34 amino acid truncation at the C-terminus of the protein. With the ligand and receptor identified, a key series of experiments led to the forging of a firm connection between the ERBB family and cancer. The first link between EGFR and tumorigenesis was the observation that EGFR shared an important functional property with the first oncogene, v-Src; it induced tyrosine phosphorylation both of itself and other cellular proteins, a property known to be essential for the transforming ability of v-Src (Cooper and Hunter, 1981; Erikson et al., 1981; Hunter and Cooper, 1981). Secondly, southern blotting demonstrated that *EGFR* was amplified in A431 cells (Ullrich et al., 1984), and both amplified and rearranged in a fraction of glioblastomas known to overexpress the receptor at the protein level (Libermann et al., 1985; Ullrich et al., 1984). These studies represent the first demonstration of aberrant growth factor receptor expression in human tumors. Thirdly, stimulation of A431 cells with EGF elicited an

increase in phosphotyrosine levels beyond that previously detected in untransformed fibroblasts (Hunter and Cooper, 1981). This association between amplified EGFR levels and increased activity, along with work from the Hunter lab confirming that tyrosine kinase activity was essential for the transforming ability of SRC (Sefton et al., 1980), cemented the connection between elevated kinase activity, increased cellular tyrosine phosphorylation, and transformation. Finally, the probing of cDNA libraries for sequences homologous to *EGFR* led to the identification of *ERBB2* (Coussens et al., 1985), subsequently found to have homology to the *Neu* oncogene, itself identified from NIH3T3 foci induced with DNA from ENU mutagenesis derived rat neuroblastomas (Schechter et al., 1985; Schechter et al., 1984). The subsequent identification of increased ERBB2 levels in breast and ovarian cancer (Slamon et al., 1987; Slamon et al., 1989) expanded the ERBB/cancer connection to other family members.

These initial experiments laid the foundation for an investigation into the role of the ERBB family in cancer and for the subsequent development of ERBB targeted therapeutics. This branch of ERBB biology has drawn heavily from work done in parallel to characterize the individual members of the ERBB family, both ligands and receptors, from both a structural and signaling perspective, and to elucidate their role in normal physiologic processes and development.

Vertebrate ERBB family expansion

The ERBB family was no exception to the RTK expansion that occurred during the evolution of vertebrates. *C.elegans* has only one receptor, *LET-23*, and one ligand, *LIN-3*, orthologs of *EGFR* and *EGF* respectively, and they are primarily involved in vulval cell fate specification (Aroian et al., 1990). *D.melanogaster* retains one receptor, *DER*, orthologous to *EGFR*, but has expanded its repertoire of ligands to five, three of which (*Spitz*, *Gurken*, and *Keren*) share homology with the vertebrate ligand *TGF-α*; one, *Vein*, with *Neuregulin* (NRG); and one inhibitory ligand, *Argos*, which has no known ortholog in vertebrates (Yarden and

Sliwkowski, 2001). The presence of only a single receptor in invertebrates limits signaling diversity, on both a qualitative and quantitative scale, primarily due to the absence of heterodimers and the resulting decrease in the spectrum of adaptor proteins that can be recruited to activated receptor molecules.

The vertebrate genome encodes four receptors, *EGFR, ERBB2/HER2, ERBB3/HER3, ERBB4/HER4.* On the basis of sequence homology it is posited that the four receptors evolved as a result of two rounds of gene duplication, the first generating the *ERBB1/ERBB2* and the *ERBB3/ERBB4* ancestral genes, followed by a second duplication to generate the canonical four family members. Only two of the four receptors, EGFR and ERBB4, are functionally autonomous; ERBB2 has no known ligand and ERBB3 is kinase-dead, relegating both to a strict dependence on other family members for full activity. The family members share a common structure, consisting of a glycosylated extracellular region composed of four domains, two of which are cysteine-rich, a transmembrane domain, a juxtamembrane domain, and a kinase domain followed by a C-terminal region harboring tyrosines, which upon phosphorylation lead to the recruitment of adaptor proteins.

ERBB family ligands have expanded in vertebrates in parallel with the receptor expansion, and to date there are thirteen known members. They can be classified into three groups based on binding specificity, (i) those that bind only ERBB1 (TGF-α, EGF, AMPHIREGULIN), (ii) those that bind ERBB1/ERBB4 heterodimers (EPIREGULIN, HB-EGF, BETA-CELLULIN), and (iii) NEUREGULINS (one subgroup is ERBB3/ERBB4 specific (NRG-1, NRG-2), and the other is restricted to ERBB4 (NRG-3, NRG-4)). In all organisms ERBB ligands exist as transmembrane precursors that require cleavage for release as soluble factors. The pro-ligand form is generally composed of Ig-like domains, glycosylated linkers, binding sites for heparin, along with an EGF-like domain that is released upon cleavage, instigated either by serine-proteases known as Rhomboids in invertebrates, or by zinc metalloproteases, ADAMs, in vertebrates.

The vertebrate expansion of the ERBB family does not merely represent an increase in size from a single mostly linear pathway of invertebrates to four such pathways. Rather, the expansion has resulted in a complex, interconnected network due primarily to the ability of the receptors to homo- or hetero-dimerize, and their differential recruitment of adaptors and ligand-binding specificity. The combinatorial nature of the ERBB family, and the resultant diversity of potential signaling modules, establishes a network topology that may be better able to sustain and adapt to mutations or alterations in individual components of the network when compared to the fragile linear pathway found in invertebrates. The specialized functions of each receptor or ligand, and the ability of the vertebrate network to tolerate lesions as a result of this redundancy, have been chiefly elucidated through the generation of null alleles in the mouse.

ERBB ligand/receptor knockout mouse models

A vulvaless phenotype in *LET-23* mutant worms (Aroian and Sternberg, 1991) along with a range of embryonic phenotypes in *DER* mutant flies, including anterior structure and nervous system defects (Schejter and Shilo, 1989), raised the question of what phenotypes, if any, would result from the deletion of *ERBB* receptors or ligands in vertebrates. Unlike invertebrates, where the pathway is mostly linear and hence the deletion of any component was expected to result in a phenotype, the vertebrate expansion of the ERBB family and ligands might have elicited increased redundancy, or an increased restriction in the role of individual ligands or receptors; hence an uncertainty surrounded the potential phenotype of knockout mice. *Egfr* knockout mice are lethal over a range of stages depending on the strain background, from embryonic day 11.5 (E11.5) (129/Sv) or P0 (C57BL/6) on standard inbred strains, to postnatal day 20 (CD1, MF1, C3H) on a more outbred background (Sibilia and Wagner, 1995; Threadgill et al., 1995). They die from a series of proliferation and differentiation defects in epithelial tissues including the placenta, lung, skin, hair, eyes, and the gastrointestinal system, and develop postnatal neurodegeneration, all confirming a role for Egfr in normal development.

Conditional knockout alleles of *Egfr* have recently been generated (Lee and Threadgill, 2009; Natarajan et al., 2007) and have elucidated a role for the receptor in proliferation of hepatocytes after liver injury. *ErbB2/ErbB4/Nrg-1* knockout mice are embryonic lethal at E10.5 due to cardiac defects, specifically an absence of trabeculae (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995), while *ErbB3* knockouts die at E13.5 due to defects in the sympathetic nervous system resulting from an absence of neural crest cell migration (Riethmacher et al., 1997).

In contrast to the severe phenotypes of the individual ERBB receptor knockout mice, milder phenotypes, generally restricted to a specific tissue, have resulted from the knockout of *ErbB* ligands, including *Tgf-α, Egf, amphiregulin* and *HB-Egf* (Iwamoto et al., 2003; Luetteke et al., 1999; Luetteke et al., 1993; Mann et al., 1993). All are viable, including the triple knockout of *Tgf-α, Egf*, and *amphiregulin*, while *HB-Egf* null mice die postnatally from cardiac defects, but with only a 50% penetrance. This striking difference in the essential nature of ligands and receptors may simply reflect the larger number of ligands, whose overlapping functional elements account for this increased level of redundancy. The availability of conditional alleles for receptors and ligands now affords the possibility of examining the effects of combinatorial knockouts on normal physiology and development, further enhancing our knowledge as to the benefits and potential vulnerabilities of the dense, interconnected ERBB network in vertebrates.

Downstream signaling pathways

Initial studies found that cells stimulated with EGF showed a robust increase in overall tyrosine phosphorylation in the cell (Hunter and Cooper, 1981), but the mechanism by which this phosphorylation of the receptor was propagated downstream and ultimately converted into a response, such as proliferation or migration, was unknown. EGF stimulation of NIH3T3 cells was found to increase GTP-bound c-H-Ras (Kamata and Feramisco, 1984), and the subsequent use of neutralizing antibodies against Ras was found to impair the ability of EGFR to transform

NIH3T3s (Smith et al., 1986), linking EGFR activation to RAS pathway activity. The first direct connection between EGFR and a cytoplasmic signaling protein was made when EGF stimulation of cells was found to promote PLC-γ phosphorylation in an EGFR kinase dependent manner and PLC-γ was found to co-immunoprecipitate with the receptor (Margolis et al., 1989). Subsequent work led to the identification of SH2 and PTB domains as the principal mediators of effector protein binding to phosphorylated tyrosine residues on ERBB receptors. The myriad tyrosines available on the four ERBB receptors bestows an astonishing degree of combinatorial complexity on the network with regard to the downstream signaling pathways that can be engaged with various combinations of homo- and hetero-dimers. ERBB3 signals almost exclusively through the PI3K pathway due to the presence of six p85 binding sites in the C-terminus; hence is often a preferred binding partner for ERBB1 and ERBB2 which could otherwise only couple indirectly to this pathway. Individual ligands can induce differential phosphorylation of a receptor and as a consequence elicit distinct recruitment of downstream signaling pathways (Saito et al., 2004). The ligands can also affect the rate and type of endocytosis and receptor recycling (Reddy et al., 1998), but the contribution of other elements of the receptor/ligand interaction to the induction of specific downstream signaling events awaits further analysis. A phosphoproteomic analysis of cells expressing EGFRvIII, a mutant receptor found in glioblastoma, has revealed that phosphorylation of individual tyrosines can affect the relative phosphorylation of other tyrosine residues in the receptor, and this is functionally propagated to downstream signaling pathways (Huang et al., 2010). This hitherto uncharacterized interdependence of tyrosine phosphorylation, in concert with improved techniques to accurately measure phosphorylation dynamics of the receptors over short time scales, challenge future research to incorporate these additional facets of ERBB signaling dynamics in their analysis.

Insights into ERBB function from crystal structures

These past eight years have been witness to a series of seminal structural papers on the ERBB family, with a focus on ligand binding, kinase domain activation and the juxtamembrane (JM) domain. These studies have had a profound impact on our mechanistic understanding of how the receptor functions, and as discussed later have unequivocally influenced our understanding of how mutations may alter the activity of the receptor, along with providing new insights for therapeutic intervention.

Crystal structures have revealed that a diverse array of strategies have evolved to facilitate ligand-induced receptor dimerization. PDGF and VEGF are monovalent ligands but each forms a homo-dimer to crosslink the corresponding receptor pair, while in the case of both growth hormone and EPO, the ligand is bivalent but monomeric, and thus a single ligand is simultaneously bound by two receptors (Lemmon and Schlessinger, 2010). The crystal structure of the extracellular domain of EGFR in both the absence and presence of ligand revealed yet another mode of activation (Ferguson et al., 2003; Garrett et al., 2002; Ogiso et al., 2002). In the absence of ligand, domains II and IV, the cysteine-rich domains, interact which serves (i) to shield a dimerization loop such that productive interaction with another receptor is precluded, and (ii) to hold domains I and III, the ligand-binding domains, apart such that the ligand-binding pocket fails to form. A significant rearrangement of the domains occurs upon ligand binding, and serves to juxtapose domains I and III, while relieving the interaction between domains II and IV and releasing the previously occluded dimerization arm. This mode of ligand-induced dimerization used by the ERBB family is unique in two regards, (i) ERBB ligands although bivalent, bind distant to the dimerization interface, and (ii) the dimerization is entirely receptor mediated, as opposed to the standard interplay of ligand/receptor binding at the interface. The subsequent crystal structure of the extracellular domain of ERBB2 revealed that this receptor exists in an untethered conformation, similar to that of EGFR in complex with ligand, such that the dimerization arm is constitutively exposed and domains I and III are juxtaposed with no

discernible space for ligand to bind (Garrett et al., 2003). The absence of sufficient space for a ligand may explain why ERBB2 is an orphan receptor. The structure also proposes a mechanistic explanation as to why ERBB2 is such a potent binding partner for other family members; it is poised, via exposure of the dimerization arm, for signaling. Although the dimerization arm is exposed, ERBB2 fails to efficiently form homo-dimers. The crystal structure revealed that although domain II is available for binding, a portion is not optimally aligned when compared to the structure of ligand-bound EGFR, and as such is proposed to inhibit homo-dimerization.

The activation of the kinase of many RTKs involves phosphorylation of a tyrosine in the activation loop with the resulting conformational changes activating the kinase. It was known that the mode of activation of the kinase domain of EGFR was not mediated by such a mechanism since phosphorylation of the tyrosine residue in the activation loop is not required for full kinase activity of EGFR (Gotoh et al., 1992). The molecular basis of activation was finally elucidated in 2006 when the Kuriyan group (Zhang et al., 2006) published the crystal structure of the kinase domain, in both an inactive and active conformation. It revealed that, contrary to previous reports, the kinase is auto-inhibited prior to ligand-induced dimerization, and that unexpectedly the kinase is activated through an allosteric mechanism that involves formation of an asymmetric dimer. The mode of activation is similar to that for Src and cyclin-dependent kinases (CDKs), with the C-terminal lobe of one kinase domain activating the N-terminal lobe of the other kinase domain, and it is thought that this kinase then proceeds to phosphorylate tyrosine residues in the C-terminus of the original activator kinase. Further support for this model was garnered through a series of experiments showing that the generation of mutations at the interface of the C- and N-lobes could abolish the ability of the receptor to autophosphorylate, and the co-transfection of EGFR C- and N-lobe mutants only led to activated receptor when the mutations were in different lobes.

A simple sequence alignment also revealed that among the catalytically active family members, EGFR, ERBB2, and ERBB4, there is strict conservation of residues on both sides of this interface, lending additional support for the mechanism. The kinase-dead ERBB3 shows conservation only at the C-lobe interface, consistent with it being unable to function as an activator kinase. This crystal structure lent important insight into key differences between the mode of activation of various homo- and hetero-dimers of the ERBB family, and revealed key conformational changes that occur in the shift between the inactive and active states. In the inactive state the activation loop occludes ATP binding and distances the catalytically critical C-helix precluding a key glutamate residue from forming a salt bridge with a lysine in the ATP cleft which is required for ATP coordination. Upon ligand binding the activation loop shifts position to become solvent exposed, allowing ATP to bind, the C-helix to rotate into position, and the critical salt bridge to form. As will be discussed further, from a structural perspective this resolved many questions surrounding the activating nature of a series of kinase domain mutations found in NSCLC patients (Lynch et al., 2004; Paez et al., 2004).

Finally, an activating role for the juxtamembrane region of EGFR, in contrast to the inhibitory role of this region in most other RTKs, has recently been established (Jura et al., 2009; Red Brewer et al., 2009). The crystal structure of the kinase domain along with the JM region suggests that in the asymmetric dimer, the JM of the acceptor kinase contacts the C-lobe of the donor, thus stabilizing the dimer interface. The C-terminal tails of the kinase were also found to potentially prevent this contact in the inactive form, unmasking an additional layer of regulation to prevent aberrant activation of the receptor in the absence of an appropriate signal.

The ERBB family and cancer

Early work on the characterization of transformation ability *in vitro*

ERBB family members, EGFR and ERBB2, were initially connected to cancer through comparisons with the causative agents of either virally or ENU-induced tumors (Downward et al., 1984; Schechter et al., 1985). This was followed by classical experiments in which EGFR was overexpressed, approximately 500-1000 fold, in NIH3T3 cells and found to only induce transformation in the presence of the EGF ligand (Di Fiore et al., 1987). The contribution of heterodimer formation to tumorigenesis was initially explored through the overexpression of both ERBB1 and ERBB2 in NIH3T3 cells in the presence of ligand. The combination was found to synergize in terms of transformation ability (Cohen et al., 1996; Kokai et al., 1989). A comprehensive analysis of the transformation ability of all ERBB heterodimers was carried out in a clone of NIH3T3 cells lacking any detectable endogenous receptor (Cohen et al., 1996). Heterodimers potently induced soft agar growth in the presence of ligand, with ERBB1/ERBB2 and ERBB2/ERBB3 combinations eliciting the strongest phenotype. This solidified the idea that ERBB heterodimer formation could expand the downstream signaling capabilities of individual receptors and concurrently increase their transformation potential.

Deregulated ERBB signaling in tumors

Studies of ERBB receptor signal transmission and the associated modes of regulation have revealed a number of ways in which deregulated ERBB signaling could potentially lead to oncogenic transformation. Modification of ERBB receptor signaling in tumors can generally be bracketed into alterations in (i) receptors, (ii) ligands, or (iii) mechanisms that terminate ERBB signaling.

Receptor alterations

Direct alterations at the level of the receptor have been documented to occur via an increase in expression levels, through mutation, or often a combination of both. EGFR has been found to be overexpressed in 20-90% of solid tumors (Rowinsky, 2004). This has been shown to primarily arise through amplification of *EGFR* at the genomic locus, but may also occur through an increase in the transcription of the receptor, stability of the mRNA or translation rate (Citri and Yarden, 2006). The first evidence of a functional role for ERBB receptors in human tumors was the result of pioneering work in the field of targeted therapy carried out in the mid-1980s by Dennis Slamon; he teamed up with Genentech to assess the status of *ERBB2* in human breast cancer samples (Slamon et al., 1987; Slamon et al., 1989). *ERBB2* was amplified in 30% of cases, and amplification was correlated with decreased survival, thus implicating the receptor in the pathogenesis of breast cancer. FISH positivity for *ERBB2* has since been correlated with advanced tumor grade, lymph node metastasis and the absence of steroid hormone receptors (Rowinsky, 2004). While EGFR overexpression has been documented in a range of epithelial tumors, unlike ERBB2 efforts to move beyond this correlation to evidence of a functional role for this overexpression in tumor development have met with as much success.

Until recently *EGFR* mutation had primarily been associated with glioblastomas (GBMs), where it is aberrantly expressed either through gene amplification (40% cases), mutation (20-30% cases), or both (Furnari et al., 2007). This mutation known as *EGFRvIII*, is an in-frame deletion of exons two to seven, which confers ligand-independence. Despite being a constitutively active form of the receptor, it displays significantly reduced levels of tyrosine phosphorylation than that elicited by stimulation of the wild-type receptor with EGF (Huang et al., 1997). Until the recent discovery of *EGFR* kinase domain mutations in NSCLC, *EGFRvIII* was the preeminent mutant version of *EGFR* with a documented clinical connection. A common feature of GBMs is a high degree of intratumoral heterogeneity. Recent studies have uncovered a paracrine loop between glioma cells that express EGFRvIII and those that express amplified

wild-type EGFR, with the former secreting IL-6 leading to activation of gp130 and ultimately EGFR on the latter (Inda et al., 2010). This is a novel role for mutant EGFR in actively maintaining tumor heterogeneity and driving proliferation of the bulk tumor through cytokine mediated avenues; this introduces the prospect of targeting either the receptor or the cytokines released as a means of therapeutic intervention.

Ligand alterations

As previously detailed, overexpression of wild-type ERBB receptors, even as hetero-dimers, can only transform NIH3T3 cells in the presence of ligand (Cohen et al., 1996). It is perhaps not surprising therefore that many of the tumors that overexpress the wild-type receptors usually co-overexpress at least one of the ligands (Tateishi et al., 1990). ERBB receptors have also been shown to initiate a positive feedback loop through the transcriptional induction of ligands upon stimulation with EGF, and thus receptor overexpression has the potential to initiate a self-sustaining autocrine loop. An active role for ligands in modulating tumor growth and/or resistance to therapeutics was demonstrated through the use of INCB3619, an ADAM inhibitor, on NSCLC cell lines (Zhou et al., 2006). INCB3619 prevents shedding of many ligands, including heregulin, and was used to demonstrate that many NSCLC lines rely on ADAM17 expression for signaling through ERBB receptors, specifically ERBB3, and that inhibition of ADAM17 could increase sensitivity to tyrosine kinase inhibitors. Due to the large number of ligands and their seeming redundancy based on knockout models, minimal efforts have gone into attempting to directly target them in tumors.

Alterations in ERBB signal termination mechanisms

Increased receptor/ligand levels or activity is the principal connection between RTKs and cancer. A growing body of evidence supports the concept that defective receptor downregulation mechanisms may also contribute to malignant transformation, either alone or in conjunction with the aforementioned lesions. Internalization, followed by either receptor

recycling or degradation, is an essential mechanism used to terminate RTK signaling in the event of ligand stimulation, and generally occurs through the recruitment of E3 ligases that ubiquitinate the receptor and target it for destruction. The decision between receptor recycling and lysosomal targeting is influenced in part by the ligand that binds the receptor. EGF remains stably associated with EGFR upon internalization into endosomes, thus marking the receptor for degradation; in contrast TGF-α dissociates from the receptor in the low pH of the endosome, allowing the receptor to exit the degradation pathway and be recycled to the surface (French et al., 1995; Reddy et al., 1998). The ERBB family composition of a cell can also influence receptor degradation. EGFR is the only family member that internalizes at an appreciable rate, and the only member that recruits an E3 ligase, Cbl, to a phosphotyrosine site upon ligand stimulation (Baulida et al., 1996); although a recent study demonstrated that a conserved Cbl-binding site found in both ERBB2 and ERRB4 is in fact functional when inserted into EGFR (Jansen et al., 2009). This striking difference in signaling attenuation mechanisms between the ERBB family members lends greater significance to the formation of EGFR heterodimers. These receptor pairs will internalize at a lower rate than EGFR homo-dimers and potentiate ligand-induced signaling; another example of how the expansion in receptor/ligand numbers in vertebrates have dramatically altered the signaling capability of the ERBB family.

ERBB targeted E3 ligases are a major component of the mechanisms cells use to terminate receptor signaling. c-CBL, the principal E3 ligase known to target EGFR, has a PTB domain and binds either directly to EGFR-p-Y1045 or indirectly via GRB2 at p-Y1068, resulting in receptor ubiquitination. Monoubiquitination is proposed to mediate internalization, while polyubiquitination is involved in lysosomal targeting (Haglund et al., 2003; Huang et al., 2007). CBL was originally thought to be an oncogene due to its homology with v-CBL, isolated from a retrovirus that induced B-cell lymphoma and leukemia in mice. This was resolved when v-CBL was found to act as a dominant negative as it has a truncation of the RING domain but retains the ability to bind phosphotyrosine (Mosesson et al., 2008). A role for CBL in downregulation of

EGFR was first elucidated through the study of a loss-of-function mutant of the *C.elegans* homolog, *SLI-1*, which was found to act as a suppressor of a hypomorphic allele of *LET-23* (Yoon et al., 1995). Further studies went on to characterize binding of CBL to EGFR and its role in receptor ubiquitination (Galisteo et al., 1995). A number of oncogenic RTKs have been shown to have defects in CBL-mediated receptor downregulation including EGFRvIII, v-erbB, TPR-MET, and v-kit (Peschard and Park, 2003), and conversion of Y1045F in EGFRvIII decreases receptor degradation, increases recycling, and enhances transformation ability (Davies et al., 2006). There are conflicting reports as to the role of CBL in regulating kinase domain mutants of EGFR found in lung cancer. These EGFR mutants are internalized at a decreased rate compared to wild-type (Hendriks et al., 2006), and in one context in response to TGF-α there was diminished phosphorylation of Y1045, decreased phosphorylation of CBL, reduced CIN85 recruitment, but a high basal CBL/EGFR association (Shtiegman et al., 2007; Yang et al., 2006). CIN85 is thought to cause clustering of CBL bound to EGFR to aid in internalization. Others have found the receptor to be phosphorylated at Y1045 and ubiquitinated (Han et al., 2006). The different cellular contexts in which these experiments were carried out and the assays used are likely to be responsible for the divergent results.

As exemplified above the multiple mechanisms by which ERBB signaling can be deregulated necessitates numerous layers of feedback control to contain and regulate these potent signaling molecules. It is evident that the balance between the activation and termination of signaling is likely to be disrupted in tumorigenesis. The overall output of EGFR signaling is determined by a myriad of factors, including ERBB receptor levels, ligand type and expression level, effector protein distribution, the presence of feedback molecules, the duration of phosphorylation *etc.*; as such accurate predictions as to the effect of any individual modification can only be made by taking all of these factors into consideration.

ERBB receptors and the hallmarks of cancer

Weinberg and Hanahan proposed the original six hallmarks of cancer: (1) insensitivity to anti-growth signals, (2) growth factor autonomy, (3) evasion of apoptosis, (4) sustained angiogenesis, (5) limitless replicative potential, and (6) tissue invasion and metastasis (Hanahan and Weinberg, 2000). Alterations in ERBB family members have been reported to contribute, either directly or indirectly, to all the original hallmarks apart from limitless replicative potential. While this may not be surprising given both the pleiotropic roles of the ERBB family in a multitude of cellular processes and the number of signaling pathways that are engaged through receptor activation, it underscores the possibility that the targeting of ERBB family members in tumors driven by these oncogenes might have significant therapeutic effects.

Oncogene addiction and the rationale for ERBB targeted therapy

'Oncogene addiction' was a term coined to describe the reliance of some tumors on continued signaling through a single gene or pathway for tumor cell survival and proliferation (Weinstein, 2002). On the basis of oncogene addiction the current approach of targeted therapies in the treatment of cancer seems obvious. Initial efforts to target the ERBB family began almost twenty years prior to the term 'oncogene addiction' being conceived, and as such are remarkable in their foresight. The multistep nature of tumorigenesis, the role of genomic instability in tumor progression, and the fact that tumors often evolve over long periods of time, acquiring genetic and epigenetic alterations, make it all the more remarkable that tumors can remain reliant on single lesions for their survival.

While the exact nature of oncogene addiction and the mechanistic underpinnings of the varied response of different tumor types to oncogene inhibition, from senescence, to differentiation, or apoptosis, remain to be understood, there is now evidence from a multitude of systems that this may be a universal feature of many tumor types. Studies in human cancer cell lines, initially using antisense oligonucleotides and subsequently using RNAi, found that

inhibition of the target oncoprotein abrogated the transformed phenotype, assayed by growth in soft agar, and often resulted in apoptosis (Brummelkamp et al., 2002; Colomer et al., 1994; Zhou et al., 1995). As expected there have been a number of studies on *EGFR* mutant and *B-RAF* mutant cell lines in recent years confirming data from the clinic of the dependence of tumor cells on continued expression of these proteins (Hingorani et al., 2003; Sordella et al., 2004; Tsai et al., 2008). Work in human cell lines has been complemented by, and expanded upon, by work done in genetically engineered mouse models (GEMs), beginning with the study of mutant *H-Ras* driven melanoma and *Myc* driven T-cell lymphoma and AML (Chin et al., 1999; Felsher and Bishop, 1999), followed by subsequent work in *HER2* driven breast cancer (Moody et al., 2002), and *K-Ras*^{G12D} driven lung cancer (Fisher et al., 2001) among others. While the data from doxycycline-inducible GEMs lend substantial support to the oncogene addiction hypothesis, these tumors are driven from their initial stages by high-level expression of oncogenes, a situation not expected to be mirrored in human tumors. It is therefore perhaps not so surprising that these models are dependent on the continued expression of the oncogene for survival. Until a similar assessment of the dependence of tumors that arise in models where the oncogenic lesion is at the endogenous locus, the issue of whether the doxycycline-inducible GEMs overestimate the seemingly universal dependence of tumors on a single oncogenic lesion remains.

The most relevant data to support oncogene addiction comes from clinical trials involving targeted therapies. The initial development of Herceptin for ERBB2 overexpressing breast cancer (Slamon et al., 2001) and Gleevec for CML (Druker et al., 2001a; Druker et al., 2001b) has been followed by success targeting EGFR in NSCLC (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004) and B-RAF in melanoma (Bollag et al., 2010; Flaherty et al., 2010). The success of Gleevec is astonishing considering that in the early 1990s, when the idea of targeting BCR-ABL was initiated, it was unknown whether tumors retained any dependence on the initiating lesion, and whether the multitude of other changes that had occurred during tumor

evolution would necessitate the targeting of many proteins to elicit a response. Pioneering work on the dependence of BCR-ABL mutant leukemia cells on kinase activity and the abrogation of colony growth in the presence of inhibitor (Buchdunger et al., 1996; Druker et al., 1996) led to a clinical trial, the results of which have had profound implications, both for how we think about tumor evolution and the current approach to cancer therapies. The observed cytogenetic remissions of patients, not only in chronic but also in blast crisis phase, served as the basis for the idea of 'oncogene addiction', and this surprising result, given the supposed complex nature of late stage tumors, has fuelled efforts to define the driver mutations of all human tumors with the expectation that their inhibition may lead to similarly remarkable responses. The field of targeted therapy/oncogene addiction has evolved along a circuitous route and has taken advantage of data from a range of systems. It is interesting to note that phase 1 trials of Gleevec began with minimal data from human cell lines and before there was any data using GEMs to support such an idea as oncogene addiction, and in many respects the targeting of EGFR in NSCLC has followed a similar route.

Therapeutic targeting of the ERBB family

The rationale for therapeutic targeting of the ERBB family was that the receptors and ligands are overexpressed in many different types of tumors and such overexpression has been correlated with poor prognosis. The benefits to targeting RTKs are that (i) they are druggable, and (ii) they are accessible to either antibodies that target the extracellular domain or small molecules against the kinase domain. Initial work uncovering the link between *ERBB2* amplification and decreased survival rates in breast cancer patients led to the development of trastuzumab (Herceptin), a monoclonal humanized antibody targeting ERBB2, shown initially to inhibit ERBB2 mediated transformation of NIH3T3s (Hudziak et al., 1989). Although the exact mechanism of action is still contentious, Herceptin has been shown to bind to domain IV of the extracellular region of the receptor, leading to internalization and degradation. It is now in use as

a single agent in frontline therapy for metastatic breast cancer found to be FISH positive for *ERBB2* (Mass et al., 2005). The timeline between the discovery of *ERBB2* amplification in breast cancer patients and the approval of Herceptin for use in the clinic is unparalleled. Herceptin represents the first targeted therapy for cancer, and laid the foundation for future efforts to target other kinases.

Monoclonal antibodies (mAbs) against EGFR

On the basis of the overexpression of the ERBB receptors and ligands in tumors, along with data purporting to demonstrate a role for antibodies in modulating receptor proteins in various autoimmune diseases (Wilkin, 1990), Mendelsohn and colleagues set out to develop a series of antibodies against EGFR using A431 cells as an immunogen (Sato et al., 1983). mAb225 was one of the antibodies identified that inhibited proliferation of A431 cells, both in culture and in xenografts (Gill et al., 1984; Masui et al., 1984; Sato et al., 1983). It acts both through inhibition of EGF binding and induction of receptor downregulation. Clinical trials with C225 began in 1991 (Divgi et al., 1991), and have led to the use of cetuximab, the chimeric version of mAb225, to treat irinotecan-refractory metastatic colorectal cancer. While the timeline to transition antibodies targeting EGFR to the clinic has trailed that of Herceptin, the proposal to target the receptor using antibodies was made in the early days of research into the role of EGFR in tumors (Mendelsohn, 1990). Future work on antibody-mediated therapy against EGFR will require novel biomarkers that will identify tumors dependent on EGFR signaling and ideally be predictive of patient response, in a manner analogous to the response of EGFR mutant NSCLC to tyrosine kinase inhibitors.

Tyrosine kinase inhibitors (TKIs) against EGFR

Due to their surface accessibility antibody-mediated therapy was the initial approach taken to target ERBB family members. This was followed by efforts in the late 1980s and early 1990s to identify small molecule kinase inhibitors that would be cell permeable and orally active.

These two features, in conjunction with the potential of a TKI to target ligand-dependent and independent receptors as well as constitutively active kinases, led to a shift in the focus of EGFR targeted therapy, from antibodies to small molecules. The foundation for developing EGFR kinase inhibitors was centered around two main findings, (i) kinase activity of TKs had been shown to be essential for transformation, and (ii) mutation of a residue in EGFR essential for ATP binding, K721A, abrogated transformation (Honegger et al., 1987a; Honegger et al., 1987b; Redemann et al., 1992). Structural studies of PKA and other kinases had revealed that the ATP binding cleft of TKs is highly conserved, even amongst evolutionarily distant kinases; a major concern that then arose was whether individual kinases could be specifically targeted, and whether inhibitors could be identified that could compete with the high concentration of intracellular ATP. The screening of libraries of compounds against the wild-type EGFR identified a subset of compounds, known as 4-anilinoquinazolines, a class of ATP competitive inhibitors, with IC_{50} values in the submicromolar range (Fry et al., 1994; Wakeling et al., 1996; Yaish et al., 1988). This led to the development of two TKIs, gefitinib (Iressa) and erlotinib (Tarceva), which are reversible ATP-competitive inhibitors designed originally to target wild-type EGFR. The use of these compounds in NSCLC patients led to the fortuitous discovery of EGFR kinase domain mutations in a subset of NSCLC patients that were predictive of therapeutic response.

EGFR mutations in NSCLC

Initial discovery and characterization

Gefitinib and erlotinib were selected for use in NSCLC due to the overexpression of EGFR in over 60% of cases and the correlation of receptor expression with poor prognosis (Hirsch et al., 2003; Nicholson et al., 2001; Ohsaki et al., 2000). Partial radiographic responses were seen in 10% and 28% of cases in phase II trials of NSCLC patients refractory to chemotherapy, in the US and Japan respectively (Fukuoka et al., 2003; Kris et al., 2003). A

number of attempts to correlate response to levels of the proposed target, EGFR, or activity of any of the downstream signaling pathways failed, mirroring original findings from cell lines (Magne et al., 2002; Suzuki et al., 2003; Wakeling et al., 2002). Responses were found to correlate with a subgroup of patients displaying some or all off the following clinical features: Asian ethnicity, female gender, adenocarcinoma subtype, and never smokers (Miller et al., 2004). In contrast to the approval of imatinib for CML, the first targeted therapy for chemorefractory NSCLC was approved despite a lack of understanding as to what factors determined response.

The dramatic and rapid responses of a fraction of patients led three groups to probe the underlying molecular mechanism further, either through sequencing the *EGFR* coding sequence from patients that had responded to gefitinib (Lynch et al., 2004; Pao et al., 2004), or by sequencing the exons encoding the activation loop of 47 RTKs in 58 NSCLC samples, none of which had been treated with gefitinib (Paez et al., 2004). The results found a striking correlation between the presence of mutations in the kinase domain of *EGFR* and response to TKIs, either gefitinib or erlotinib. EGFR mutant NSCLC patients have been found to have a better prognosis than patients whose tumors harbor wild-type EGFR or mutant K-RAS, independent of treatment with TKIs, and in conjunction with their dependence on EGFR signaling this has resulted in their designation as a specific subgroup of adenocarcinoma patients (Eberhard et al., 2005).

A series of prospective single-arm studies have since documented radiographic response rates of 55-91% in patients with *EGFR* mutations treated with TKIs (Pao and Chmielecki, 2010). Two prospective phase III trials have also demonstrated that first-line treatment with TKI is more beneficial than chemotherapy for patients with EGFR mutant lung tumors (Maemondo et al., 2010; Mitsudomi et al., 2010; Mok et al., 2009; Rosell et al., 2009). The demographic of patients whose tumors harbor EGFR mutations has since been found to coincide with that of patients found to respond to TKIs (Janne and Johnson, 2006; Miller et al.,

2004). Although the mutation frequency differs between the US/Europe and East Asia, the response rate of patients with mutations is similar, at around 75% (Tsao et al., 2005).

The mutations are found in the kinase domain, with either small in-frame deletions in exon 19 (Del19) or a point mutation in exon 21 (L858R), accounting for approximately 44% and 41% of all cases respectively (Gazdar, 2009). The deletions are in the N-lobe, while the L858R mutation is in the C-lobe of the kinase domain. These two types of mutations may not be functionally equivalent, as manifest by their differential response to TKIs. Tumors carrying deletion mutations generally show a better response to TKIs than the L858R mutant tumors, resulting in improved survival (Riely et al., 2006), but the basis for this remains unknown. Other missense mutations in exon 18 and insertion mutations/duplications in exon 20 are much less common, with the former conferring TKI sensitivity, while the latter is found to confer resistance (Greulich et al., 2005).

The initial discovery of the mutations in lung tumors and the dependence of tumors with these mutations on EGFR signaling supported the idea that these were activating mutations. This was further supported by studies in either NIH3T3 or Ba/F3 cells that found ectopic expression of the mutant receptor to be sufficient to confer a number of transforming properties on these cells (Greulich et al., 2005; Jiang et al., 2005). Despite this evidence, the location of the mutations in the known structure of the receptor failed to give much insight as to the basis for their oncogenic nature. With the revelation of the crystal structure of the TK domain, in both the inactive and active states, their means of activation became more apparent (Zhang et al., 2006). The L858R mutation is in the activation loop and this residue occupies two distinct positions depending on whether the receptor is in the inactive or active state. In the inactive state it occupies a tightly packed hydrophobic pocket and is involved in keeping the αC helix apart from key activating residues, while in the active state it is surface exposed and not in a position to influence activity of the kinase. The alteration from a small hydrophobic leucine to a polar bulky arginine residue disrupts the hydrophobic packing in the inactive state, and the

equilibrium between inactive and active states is shifted towards the latter. A similar explanation can account for the oncogenic character of the exon 19 deletion mutations. The mutations alter different parts of the kinase domain and knowledge of the structure now provides an opportunity to make novel inhibitors that are specific, and perhaps more potent, for each subset of mutations and the receptor conformation that results. While crystal structure data (Yun et al., 2007) revealed that the mutant receptors may be more susceptible to gefitinib partly due to the equilibrium shift to the active state, the mutant receptor also has a higher K_m for ATP and a lower K_i for TKIs (Carey et al., 2006; Yun et al., 2008), the balance of these effects leading to more potent inhibition of the mutant than the wild-type receptor. The availability of a crystal structure of the mutant EGFR has been informative with respect to the potential efficacy of other small molecules, for instance lapatinib, a dual EGFR/ERBB2 inhibitor that preferentially binds in the ATP cleft when the kinase is in the inactive conformation. On the basis of the crystal structure this should greatly reduce the likelihood that EGFR mutant tumors would be responsive to this drug, and limited studies so far seem to confirm this (Ross et al., 2010).

As with many oncogenes a comprehensive understanding of the mechanisms that mutant EGFR employs to elicit a transformed phenotype is currently lacking. Initial studies found that mutant EGFR strongly coupled to the AKT and STAT3/5 signaling arms (Greulich et al., 2005; Jiang et al., 2005). EGFR can only indirectly activate AKT, but while investigating gefitinib sensitivity in a series of NSCLC cell lines, ERBB3, with its six p85 binding sites, was shown to be the primary mediator of AKT activation in the context of mutant EGFR and also essential for viability of these lines. The implication of multiple members of the ERBB family in dictating response expands the oncogene addiction model. ERBB2 expression has also been implicated in response to TKIs (Cappuzzo et al., 2005), however more work needs to be done to confirm a functional rather than a biomarker role for HER2 signaling in mutant EGFR driven tumorigenesis.

Amplification in EGFR mutant NSCLC

EGFR TKIs were initially designated for use in NSCLC based on receptor overexpression in a large percentage of tumors. While initial attempts to correlate response to EGFR found no connection with receptor levels as assessed by immunohistochemistry (IHC), other groups have since found some correlation between receptor amplification, as assessed by FISH, and response to TKIs in retrospective studies (Hirsch et al., 2006). Not all patients who respond to TKIs have mutations, and although the underlying mechanisms that dictate this response remain to be uncovered, EGFR amplification, independent of mutation, may be a factor (Tsao et al., 2005). Whether FISH positivity will serve as a general marker of response, or define the non-mutant responders, remains to be assessed in prospective studies. Initial studies focused on correlation of response to the presence of mutation, but later work revealed that in the majority of cases, both in cell lines and in tumors, the mutant allele is preferentially amplified (Li et al., 2008; Takano et al., 2005). A number of groups have tried to examine the order of events, mutation before amplification or vice-versa. Examination of normal bronchial epithelium adjacent to mutant tumors has found cells containing the mutation but no amplification, implicating the mutation as an early event in tumor formation (Tang et al., 2005). A more extensive analysis examining tumors, surrounding normal tissue, and metastases found that the distribution of mutation pointed to it as an early event, while EGFR amplification was not found in normal epithelium, was heterogeneously distributed in the primary tumor and was more uniform in the metastases (Tang et al., 2008), again suggesting that mutation precedes amplification and that the latter event may be involved in tumor progression. Recent studies examining the concordance of mutations in primary tumors and metastases has found that the latter don't always contain EGFR mutations (Park et al., 2009), and a sampling of primary tumors has also demonstrated significant tumor cell heterogeneity in some cases (Taniguchi et al., 2008). This is reminiscent of the heterogeneity documented in GBMs that express EGFRvIII, and warrants further investigation.

Hsp90 client kinases

Crystal structures have revealed that many oncogenic kinase mutations result in a shift in the equilibrium towards the active state. This state is usually occupied only transiently by normal proteins in the presence of an activating ligand and is typically unstable. In the case of oncogenic TKs, many are constitutively in the active state and as such require hsp90 to counter this conformational instability. Proteins commonly interact with chaperones while they are in the nascent stages of folding, and while hsp90 is not involved in folding associated with protein biogenesis, oncogenic kinases often require stable association with hsp90 to maintain stability of the mature form of the protein. Hsp90 is thought to buffer polymorphic variation in proteins, allowing variants to accumulate over evolutionary timescales in the absence of selective pressure. Hsp90 may play a similar role in tumors where the presence of unstable oncogenes may exceed the buffering capacity of the cell, revealing previously masked variation that can affect tumor cell progression (Whitesell and Lindquist, 2005).

Classic oncogenes that have been shown to require hsp90 include ERBB2 (Xu et al., 2001), B-RAF^{V600E} (Grbovic et al., 2006), BCR-ABL, v-SRC (An et al., 2000), and EGFRvIII (Lavictoire et al., 2003). Mutant EGFR is also an hsp90 client kinase and cells expressing the mutant protein are more sensitive to hsp90 inhibition using geldanamycin (GA), an ansamycin antibiotic, than cells expressing wild-type EGFR, leading to ubiquitylation and degradation of the receptor (Shimamura et al., 2005). Cells carrying the T790M resistance allele were also found to be sensitive to hsp90 inhibition and the sensitivity of both the L858R and the double mutant has been confirmed using mouse models, although the double mutant shows a diminished and only transient response *in vivo* (Regales et al., 2007; Shimamura et al., 2008). The regulation of hsp90 client kinase turnover has been proposed to involve E3 ligases not typically involved in the degradation of the wild-type protein. ERBB2, a classical hsp90 client, is degraded by the E3 ligase CHIP, but ERBB2 was found to retain sensitivity to GA induced degradation in CHIP$^{-/-}$ cells, implicating another E3 ligase in its degradation (Xu et al., 2002). The CULLIN5 (CUL5) E3

ligase has recently been shown to regulate degradation of ERBB2 and HIF1-α, both hsp90 client kinases (Ehrlich et al., 2009), and further analysis of the role of CUL5 in the degradation of other oncogenic hsp90 client kinases is warranted.

Acquired resistance to TKIs

It is now an axiom in the field of targeted therapy that treatment of tumors with TKIs will eventually lead to the emergence of resistance. The primary mechanism of resistance is often the mutation of a gatekeeper residue in the ATP binding pocket such that the inhibitor can no longer bind due to steric hindrance. This is the case for resistance to imatinib in BCR-ABL (T315I) and c-Kit (T670I) driven tumors and has led to efforts to develop second line inhibitors (Gorre et al., 2001; Tamborini et al., 2004).

The orthologous T790M mutation in EGFR accounts for almost 50% of all cases of resistance to the TKIs, gefitinib and erlotinib (Kobayashi et al., 2005; Pao et al., 2005). This mutation was initially thought to occlude gefitinib binding through steric hindrance in a manner analogous to imatinib and BCR-ABL (T315I). Cells carrying the resistance mutation were subsequently found to retain susceptibility to irreversible 4-anilinoquinazolines inhibitors, which should have similar binding properties to the reversible inhibitor, gefitinib (Kwak et al., 2005). An analysis of the kinetic properties of the T790M mutation found that the presence of this mutation in *cis* to L858R restored the K_m for ATP to that of wild-type EGFR, which coincides with a decrease in affinity for gefitinib relative to L858R alone (Yun et al., 2008). Irreversible inhibitors should be less susceptible to the shift in K_m for ATP and remain effective. An unexplained finding from this study is that T790M only alters the K_m for ATP when present in *cis* to L858R, but not in an otherwise wild-type context. Several features of T790M suggest that it is not solely a mechanism of drug resistance: 1) it has been found in tumors prior to treatment with TKIs and in a NSCLC cell line (H1975) established in the absence of TKIs (Engelman et al., 2006; Maheswaran et al., 2008), 2) a family carrying a germline T790M mutation has an increased

susceptibility to lung tumors (Bell et al., 2005), 3) in *in vitro* kinase activity assays T790M had a five-fold increase in activity over wild-type (Yun et al., 2008) and upon expression in HBECs and HEK293 cells displayed increased phosphorylation (Vikis et al., 2007), 4) transgenic mice with a doxycycline-inducible T790M allele develop lung tumors, granted with longer latency than similar models employing the classic mutations, L858R or DEL19 (Regales et al., 2007), 5) transformation of NIH3T3s was increased when T790M was in *cis* to L858R, along with the level of ligand-independence and phosphorylation of downstream effector pathways (Godin-Heymann et al., 2007), and 6) the T790M mutation often arises in the background of amplified mutant alleles but even at low copy number is sufficient to elicit resistance (Engelman et al., 2006). Amplification of T790M has recently been uncovered as a resistance mechanism to irreversible TKIs *in vitro* (Ercan et al., 2010). T790M would therefore seem to serendipitously confer drug resistance to reversible TKIs while also positively modulating the transforming ability of mutant EGFR.

In contrast to imatinib resistance, which is mediated through any number of different point mutations (Shah et al., 2002), resistance to EGFR TKIs occurs primarily through T790M. This may be due to the multiple functions of T790M as detailed above, or may reflect that imatinib binds the target kinase in the inactive state and so any mutation that alters this conformation will lead to resistance, while gefitinib/erlotinib bind the active conformation of EGFR, thus limiting the number of residues available for mutation. Apart from rare cases of resistance attributed to D761Y (Balak et al., 2006), L747S (Costa et al., 2008) or T854A (Bean et al., 2008) to date the only other documented mechanism of resistance is MET amplification (Engelman et al., 2007), which occurs in approximately 20% of cases. This confers resistance by maintaining ERBB3 mediated AKT signaling, previously shown to be critical to survival of NSCLC cell lines harboring EGFR mutations (Engelman et al., 2005), and represents a novel mechanism of therapeutic resistance wherein a kinase independent of the drug target is mobilized. MET amplification has also been found to exist prior to TKI administration, and

combination therapy using EGFR and MET inhibitors can effectively counter this resistance mechanism (Turke et al., 2010). Accumulating data on the preexisting nature of resistance mechanisms to EGFR TKIs warrants an improved understanding of EGFR tumor evolution, and of the selective advantage conferred by these mutations along with the associated basal differences between resistant tumors. Erlotinib treatment of transgenic mice expressing either the L858R or Del19 allele leads to the emergence of TKI resistance, but only a fraction of the tumors have the canonical resistance mutations, with 5/24 containing T790M, and 1/11 having MET genomic amplification (Politi et al., 2010). These models will prove to be useful tools for delineating the alternative modes of resistance that can emerge when EGFR mutant tumors are treated with TKIs. Multiple modes of tumor resistance can emerge even between a primary tumor and its metastases, complicating treatment regimens, but providing an opportunity to determine whether the surrounding local microenvironment influences the type of resistance mechanisms employed (Bean et al., 2007). All modes of resistance to EGFR TKIs maintain signaling either directly through the receptor or by subverting other kinases to signal through the same pathway; this continued dependence on ERBB family signaling for survival, despite a multitude of other alterations that probably exist, makes EGFR mutant lung cancer a quintessential example of oncogene addiction.

Response of EGFR mutant tumors to mAbs

Tumors bearing *EGFR* mutations, while sensitive to TKIs, seem to be refractory to mAb mediated therapy (Janjigian et al., 2011; Neal et al., 2010). This is consistent with data from NSCLC cell lines harboring EGFR mutations (Mukohara et al., 2005) but moderately inconsistent with data from mouse models where tumors driven by L858R are responsive to cetuximab, while those expressing Del19 are not (Ji et al., 2006a). The lack of sensitivity of mutant receptors to cetuximab may be related to the conformation of the mutant receptor or the

degree of ligand-independence, which may differ depending on the mutation and the context in which the experiment was conducted.

Tissue distribution of mutations

One of the initial papers reporting the presence of *EGFR* mutations in NSCLC also screened other tumor types for these mutations but none were found (Lynch et al., 2004). This restriction of the mutations to NSCLC has essentially held up over the past few years as many human tumor samples and cell lines have been analyzed for the presence of mutations and only rare cases, either outside of the lung (Foster et al., 2009; Kwak et al., 2006) or in the context of SCLC, have been reported (Shiao et al., 2011). This profile of restricted tissue distribution is reminiscent of *EGFRvIII*, which is detected almost exclusively in glioblastoma. The basis for the restricted profile of EGFR kinase domain mutations is unknown but could be due to certain features of the microenvironment of the lung such as ligand distribution, or if an environmental exposure is responsible for the mutations that could also limit the tissue distribution. A deeper understanding of how, or if, tissue type can modulate the oncogenic potential of these mutations would contribute to our understanding of how these mutations initiate tumorigenesis.

EGFR mutation frequency in different populations

The frequency of EGFR mutations in NSCLC is 10% in Caucasians but approaches 50% in East Asians. This distribution should spur efforts to carry out clinical trials in different populations and suggests the importance of context for these mutations to elicit an oncogenic phenotype. While the frequency of mutation differs between populations, the response to TKIs is similar, confirming that mutation is the major determinant of response (Sakurada et al., 2006; Tsao et al., 2005). In an effort to gain insight into both the apparent susceptibility of the East Asian population to NSCLC driven by *EGFR* mutations, and to understand the basis for TKI response in tumors without a mutation, three known polymorphisms, CA-SSR1 (a polymorphic dinucleotide repeat in intron 1), SNP -216, SNP -191, were examined in a large cohort of

samples, including both lung tumors and normal tissue, from a range of ethnicities (Nomura et al., 2007). The overall finding was that polymorphisms associated with increased EGFR expression were actually rare in East Asians compared to other populations, but that tumors from East Asians had allelic imbalance with one EGFR allele undergoing selective amplification, and this tended to be the allele with the shorter CA-SSR1 repeat. These findings diminish the possibility that Asian populations are more susceptible to *EGFR* mutation due to higher basal EGFR expression levels; in fact the opposite seems to be true. The selective amplification of the mutant allele is consistent with previous data on amplification in *EGFR* mutant tumors. While this may explain the occurrence of *EGFR* mutation concomitant with amplification if a threshold level of EGFR signaling is required for transformation, it fails to explain the increased frequency of *EGFR* mutations in the Asian population. In fact the data from this study would suggest that the Asian population should be less susceptible to transformation by mutation alone. A number of genome wide association studies have been carried out in an attempt to understand the basis for the increased incidence of EGFR mutant lung cancer in the Asian population, but to date have failed to identify genes that could account for this (Hsiung et al., 2010; Yoon et al., 2010).

Oncogenes are primarily activated by either mutation or overexpression mechanisms. Increasing evidence points to the existence of mutant allele specific imbalance (MASI) in many tumors harboring classic oncogenic mutations (LaFramboise et al., 2005; Soh et al., 2009). This may occur through uniparental disomy (UPD), reflected in duplication of the mutant concurrent with loss of the wild-type allele, as occurs in many *K-RAS* and *JAK2* mutant tumors. In the case of *EGFR* mutations MASI was found in approximately 75% of cases, including material from both tumor and cell line samples (Gandhi et al., 2009; Soh et al., 2009). This further supports the co-occurrence of *EGFR* amplification and mutation in NSCLC patients, but also raises questions as to the trichotomy that exists among oncogenes from *PIK3CA* where MASI is rare, to *K-RAS* where UPD is common, to mutant *EGFR* where MASI results from copy number gains (CNGs). This may reflect a difference in the threshold of oncogenic signaling required to induce

tumor formation in each case, an inhibitory role for the wild-type allele, or may reflect inherent differences in the evolution of each tumor type.

Threshold levels of oncogene expression required for transformation

The multiple hit hypothesis of tumor formation requires the activation of different signaling components and loss of tumor suppressor functions for a tumor to develop. Mutation and overexpression of the same protein, specifically a receptor, could be construed as two independent hits, as they likely have different effects on the signaling dynamics of the network, and only together may be sufficient to surpass a threshold of signaling required for oncogenesis. The threshold level of oncogenic signaling is likely to be context dependent and may contribute to the differential sensitivity of tissues to oncogenic lesions as initiating events.

Threshold requirements for oncogenes were appreciated prior to any mechanistic understanding as to their mode of action. Studies using Rat-2 cells expressing different amounts of v-Src revealed that when this oncogenic protein was expressed at low levels the cells appeared normal (Jakobovits et al., 1984). Increasing the levels by as little as four-fold had a dramatic effect and resulted in morphological changes and anchorage-independent growth. Earlier work had shown that overexpressing c-Src in Rat-2 cells at levels ten-fold above endogenous, and three-fold above the level needed for v-Src transformation, failed to elicit any properties of transformation (Parker et al., 1984). This was some of the first evidence that there might be thresholds of signaling required for oncogenic transformation, and that there may be quantitative and qualitative changes in signaling associated with crossing this threshold, the exact nature of which remains to be elucidated. c-Src has since been found to be partially transforming when expressed in *Csk* null mouse embryonic fibroblasts (MEFs) (Oneyama et al., 2008). Csk is a kinase that attaches an inhibitory phosphorylation to the C-terminus of Src, and the ratio of these proteins modulates the oncogenic potential of c-Src.

The first non-viral oncogene cloned was *RAS*, and unlike the later discovery of receptor overexpression and amplification, it was found that a single point mutation was responsible for its transforming activity (Shih and Weinberg, 1982). Mutant *K-RAS* has since been found to be amplified in many human tumors, and loss of the wild-type copy was found to increase susceptibility to chemically induced lung tumors in mice (Soh et al., 2009; Zhang et al., 2001), implicating levels of this mutant GTPase in tumor progression. A single copy of oncogenic *K-Ras* is sufficient to initiate tumor formation in mice (Jackson et al., 2001), but amplification of oncogenic *K-Ras* and loss of the wild-type copy has been recently shown to occur during lung tumor progression, and correlates with increased MAPK pathway signaling and increased sensitivity of these tumors to p53 restoration (Feldser et al., 2010; Junttila et al., 2010). Even in the case of mutant *K-Ras* it seems that threshold levels of signaling are required for progression to advanced lesions, and an understanding of the differences in signaling dynamics that occur between a single mutant allele and amplification will further our understanding of how mutant *K-Ras* tumors develop.

ERBB family members are frequently amplified or overexpressed in human tumors and how this increase in receptor levels alters signaling dynamics is incompletely understood. In an attempt to gain a quantitative understanding of adaptor protein recruitment to ERBB receptors, a protein microarray was developed containing almost all SH2 and PTB domains in the genome, hybridized with phosphopeptides from each of the family members, and binding affinity measured (Jones et al., 2006). While there are obvious caveats to this study, principally that only potential rather than actual binding partners are identified, it did reveal interesting insights into possible protein recruitment at different receptor levels. By changing the affinity threshold for interaction it was found that the number of proteins that interact with either EGFR or ERBB2 increases as the affinity threshold is lowered, mimicking the effects of increasing receptor levels. In contrast the ERBB3 interaction map changes very little over a range of binding affinities. This suggests that signaling through EGFR and ERBB2 changes on both a qualitative and

quantitative scale when receptor levels increase, for instance in a tumor. The relative importance of the quantitative and qualitative differences are unknown, but the relative absence of *ERBB3* amplification in human cancer in comparison to *EGFR* and *ERBB2* point to the recruitment of novel adaptors and engagement of different signaling pathways as playing a significant role in the oncogenic potential of overexpressed EGFR and ERBB2. The recruitment of these adaptors is highly dynamic and may depend on the local concentration of both receptor and the adaptor, and may change as the receptor is internalized and the local environment is altered (Burke et al., 2001). This needs to be considered in the context of mutant EGFR as it has been shown to internalize, at least in response to EGF at early time-points, at a lower rate than wild-type (Hendriks et al., 2006; Lazzara et al., 2010), but the impact of this on the resulting signaling dynamics is unknown.

Insights into threshold requirements from studies on Myc

The Myc oncogene is generally released from its normal regulatory controls and/or overexpressed in human tumors. The relative contribution of each to tumorigenesis could not be assessed through ectopic expression, either in cell culture or using transgenic mice, as this resulted in Myc expression no longer being under the regulation of normal cellular signals along with overexpression. This question was recently tackled using a conditional allele of *MycER* targeted to the Rosa26 locus (Murphy et al., 2008). The authors found a sharp threshold for induction of proliferation in most tissues, with the two-fold shift in levels from heterozygous *R26-MycER/+* to homozygous *R26MycER/R26MycER* demarcating this point. The induction of intrinsic tumor suppression mechanisms, such as the induction of ARF and apoptosis, required higher levels of Myc than those required to elicit proliferation. This is informative as to how cells differentiate between normal signaling and oncogenic signaling, and suggests that while low level oncogene expression may be sufficient to induce proliferation in certain tissues, high level

expression or amplification may require the subversion of the intrinsic tumor suppressor network in order to be sustained.

Coincident with thresholds of oncogene activity being required for tumor initiation and progression, there is a similar requirement for threshold levels of oncogene expression for tumor maintenance. Data to support this comes mainly from doxycycline-inducible mouse models of cancer. Most cases demonstrate a qualitative requirement for oncogene expression to maintain tumors (Chin et al., 1999; Politi et al., 2006) but a recent study tried to determine on a quantitative level how much Myc is required to maintain tumors and found that a specific threshold level indeed existed, at least in the context of a model of T-cell lymphoma (Shachaf et al., 2008).

Mouse models of cancer

Historical perspective

Mouse models of cancer have their foundation in three pioneering techniques: (i) the derivation and culturing of embryonic stem cells, (ii) the advent of homologous recombination technology in these cells, and (iii) the generation of transgenic animals through the pronuclear injection of DNA. The first modifications of the mouse genome at the organismal level involved the injection of SV40 viral DNA into mouse blastocysts (Jaenisch and Mintz, 1974), followed by transmission of Moloney murine leukemia viral sequences through the germ line (Jaenisch, 1976). The first transgenic mice that demonstrated germline transmission and that did not involve viral sequences involved pronuclear injection of either rabbit β-globin (Costantini and Lacy, 1981), thymidine kinase (Gordon and Ruddle, 1981) or human β-globin DNA (Steward et al., 1982). The chronological convergence of the discovery of oncogenes with the ability to generate transgenic mice led a number of groups to attempt to generate mice that would ectopically express an oncogene, with the expectation that just as in cultured cells,

transformation would ensue and tumors would develop. The serendipitous inclusion of the SV40 small and large T-antigens along with the SV40 enhancer and promoter sequences in an attempt to increase expression of either thymidine kinase or rat growth hormone genes led to transgenic mice that succumbed to brain tumors (Brinster et al., 1984). This was followed by the development of a more tissue specific approach, with the *Eμ-Myc* model of lymphoma (Adams et al., 1985) and the *MMTV-Myc* model of breast cancer (Stewart et al., 1984). This early work laid down important benchmarks for the cancer mouse modeling field, and established the merit in efforts to model this complex disease in whole organisms. The original models demonstrated that in principle transgenic animals expressing oncogenes in a tissue-specific manner could be generated, and while serving to confirm the causal nature of oncogenes in tumorigenesis they also supported the idea of multi-step tumor formation due to both the latency and low frequency with which tumors generally arose.

The successful derivation, subsequent culturing, and germline transmission of embryonic stem cells (Bradley et al., 1984; Evans and Kaufman, 1981; Martin, 1981) was followed by efforts to manipulate the cells in a targeted manner using homologous recombination. The principal was first demonstrated in somatic bladder carcinoma cells (Smithies et al., 1985) and this was followed by the first cases of germline transmission of targeted ES cells with modifications at the X-linked *Hprt* locus (Doetschman et al., 1987; Thomas and Capecchi, 1987). This heralded a new era in mouse modeling, as loci could now be modified in a targeted manner, endogenous promoters could be used to drive expression of reporter alleles, and deletions of tumor suppressor genes now became a possibility. The first report of a cancer prone mouse generated using homologous recombination followed in 1992 with the publication of the *Rb* heterozygous knockout mouse that developed pituitary tumors (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992), followed by the fortuitous selection of *p53* as a gene to knockout since it is viable as a knockout yet susceptible to tumors, in this case lymphomas and sarcomas (Donehower et al., 1992; Jacks et al., 1994).

Pioneering mouse models

Rather than describe the vast array of tools that can be currently deployed to model tumors in the mouse I have opted to select some of the truly pioneering models in the field, all of which significantly impacted the way subsequent modeling was done.

The first transgenic mice expressing oncogenes (Adams et al., 1985; Brinster et al., 1984) and the first mice that were homozygous null for a tumor suppressor (Donehower et al., 1992; Jacks et al., 1994) were seminal events in the field as they established beyond any doubt the causal nature of oncogenic and tumor suppressor lesions. Some of the key challenges facing the modeling community in the early 1980s were that at the time most cancer mouse models were either spontaneously arising or carcinogen-induced. The obvious caveats are the lack of control over the timing and location of tumors, of the type of mutations induced, and of the number of tumors arising. While this led to a reliance on xenografts and orthotopic models, advances in transgenic technology and gene targeting provided sufficient impetus to attempt to develop improved autochthonous models. Some of the key features of human tumors that would ideally be recapitulated in all mouse models are 1) that tumors arise somatically and in a sporadic fashion, surrounded by normal host cells and a functioning vascular and immune system, 2) that the expression of oncogenes does not occur at supraphysiological levels, 3) that the lesions modeled, both in target and type, reflect the type of mutations found in the equivalent human tumors, 4) that the number of tumors arising in a given tissue is low and the mutations are initially expressed in only a handful of cells and not an entire tissue or animal, and 5) that tumors evolve in a multistep process such that mutations should be induced sequentially rather than concurrently. Most of these targets have been met to some extent in many of the models currently in use, however others remain challenges for the field, and the incorporation of as many of these features as possible is essential if mouse models of cancer are to live up to their potential to contribute to the development of a cure for the disease.

Efforts to model oncogenic and tumor suppressor lesions have met with different obstacles due to the nature of the lesion that needs to be engineered. Proto-oncogenes are almost universally important during development due to their varied roles in proliferation, differentiation, migration and apoptosis, and hence deletion or inactivation of such genes is often lethal (Johnson et al., 1997; Threadgill et al., 1995), while constitutive whole animal, and often even tissue restricted expression, is also usually lethal if it occurs during development (Tuveson et al., 2004). The characterization of tissue-specific promoters and their juxtaposition to cDNAs of interest was the first step in countering the embryonic lethality or unrestricted expression associated with a standard constitutively expressed transgene. Tumors, apart from those connected to germline predisposition syndromes, arise spontaneously in postnatal somatic tissues and this represented perhaps the greatest challenge facing the modeling community – how could models be developed where oncogenes could be activated or tumor suppressors deleted in a temporally and spatially controlled fashion. The issue was more pressing for the tumor suppressor aficionados as tumor suppressor knockouts are either embryonic lethal, precluding the examination of null cells in adult mice apart from those studied in the context of chimeras (Cichowski et al., 1999; Williams et al., 1994), or develop a broad range of tumors types which may result in death before the desired phenotype emerges.

Doxycycline-inducible alleles

The combination of doxycycline regulatable cDNAs with first tTA and later rtTA transactivators produced mice that allowed conditional expression of oncogenes that could also be combined with a tissue specific promoter driving the tTA/rtTA to further refine the expression pattern (Gossen et al., 1993). A parallel approach involved the use of oncogene-ER fusion proteins, which result in the heat shock protein mediated cytoplasmic sequestration and inactivation of the oncogenic protein until the addition of tamoxifen (Eilers et al., 1989). A key feature of both systems was the reversible nature of oncogene expression/activation;

accordingly, this stimulated the first attempts to test the oncogene addiction hypothesis *in vivo* and discern whether continued oncogene expression was required for tumor maintenance (Chin et al., 1999; Felsher and Bishop, 1999).

Cre-lox technology

The need to conditionally inactivate tumor suppressor genes spurred a revolution in modeling with the adoption of Cre-lox technology. The Cre-lox recombination system was adapted from phage and shown to work first in mammalian cells (Sauer and Henderson, 1989) and then in *in vivo* (Orban et al., 1992). The approach allows tissue specific disruption of tumor suppressors genes by flanking key exons at the endogenous locus with loxP sites in a manner that does not disrupt normal expression of the gene. These alleles are then combined with either tissue-specific Cre alleles or with the administration of viruses expressing Cre recombinase (Shibata et al., 1997); this advance permitted previously unattainable spatial and temporal control over targeted modifications in the genome. This technological advance was layered on top of the doxycycline and tamoxifen inducible systems with the generation of either doxycycline-regulatable Cre alleles or a CreER fusion protein, thus spatially and temporally constraining Cre activity, and indirectly the desired genomic modifications (Feil et al., 1996; Metzger et al., 1995).

The Rosa26 locus and Flp-in alleles

In an effort to counteract the problems inherent with classical transgenics, in which despite the use of tissue specific promoters the expression pattern was too often determined by the site of integration, and to allow the generation of reporter alleles with ubiquitous expression, a locus was needed that was ubiquitously expressed and whose disruption did not result in a phenotype. Through retroviral gene-trapping in ES cells the *ROSA26* locus was identified (Zambrowicz et al., 1997) and this has remained the locus of choice for targeted transgenics. This has recently been expanded to include the *ColA1* locus (Beard et al., 2006). The *ColA1*

locus does not result in ubiquitous expression of transgenes but it has the added advantage that the 3'UTR has been modified to include a FRT site. This allows constructs containing a compatible FRT site to be recombined into this locus, leading to the highly efficient generation of a single copy integrant at a defined position. This approach is not limited to the *ColA1* locus but could be employed anywhere in the genome.

Conditional expression of oncogenic lesions

Despite advances in the design and regulation of transgenic alleles, an alternative approach was needed that would mirror the situation with floxed tumor suppressor alleles and allow for conditional point mutations to be engineered at endogenous proto-oncogenic loci. The goal was both to more accurately mimic human disease and to carefully dissect out the relative contribution of mutation and overexpression to tumor initiation and progression. The first effort towards this involved an elegant model of NSCLC driven by oncogenic K-Ras (Johnson et al., 2001). The authors adopted a 'hit-and-run' strategy, previously employed as a targeting method (Hasty et al., 1991; Valancius and Smithies, 1991), to transition from a 'latent' oncogenic allele containing a duplication of exon 1 that harbors the point mutation to a restored active allele, and which is proposed to occur through spontaneous intrachromosomal recombination. The result is that the mice develop multi-focal lung adenocarcinoma with 100% penetrance. While this represents the first example of a spontaneously activating targeted mutation at an endogenous locus there are a number of caveats associated with it: the tumors arise spontaneously and asynchronously due to an inability to regulate location, timing, or incidence of the recombination event, and as a result the mice die early from an overwhelming lung tumor burden, precluding efforts to study tumor progression. These issues, along with other advances in the field, have probably precluded the development of such 'latent' alleles for other oncogenic lesions.

This next advance single-handedly revolutionized the branch of mouse modeling focused on oncogenic lesions, including both the targeting of endogenous loci and the

generation of transgenic alleles. To gain control over the number of lesions induced while retaining control over the timing and location of initiation, a lox-stop-lox cassette (LSL) was designed and inserted upstream of exon 1 of *K-Ras* that contains the mutation encoding G12D. This cassette acts as a transcriptional and translational stop element preventing expression of the mutant protein until administration of Cre recombinase deletes it leading to expression of oncogenic K-Ras (Jackson et al., 2001; Tuveson et al., 2004). The advances contributed by the use of this LSL-cassette include: (i) the synchronous induction of initiation mutations, (ii) the ability to control tumor multiplicity through titration of Cre, (iii) the conditional oncogenic allele can be paired with conditional alleles of tumor suppressors to induce combinations of lesions in the same cell while the rest of the tissue remains wild-type, and (iv) a single LSL allele can be generated but through pairing with tissue specific or inducible Cre alleles the effect of the mutation in a range of tissues can be monitored. This cassette has been used extensively both in the context of endogenous loci and also in transgenic alleles to regulate target gene expression.

Using a combination of the above approaches a number of elegant publications have modeled the majority of lesions known to occur in human cancers, from point mutations and overexpression, to deletions, translocations (Buchholz et al., 2000; Collins et al., 2000; Forster et al., 2003) and duplications (Nakatani et al., 2009; Walz et al., 2003). The generation of models that either result in a series of mutations occurring in a sequential manner or that mimic gene amplification are some of the remaining challenges facing the cancer mouse modeling community.

Technological improvements to allele generation

Several recent technological advances have influenced the approaches taken to modeling lesions and the rapidity with which they can be engineered, but two in particular merit mentioning, recombineering (Copeland et al., 2001) and zinc-finger nucleases (Porteus and

Carroll, 2005). The major contribution of both technologies is to approach a point where modifications of the genome are almost seamless, thus mirroring the human disease as closely as possible.

ERBB family mouse models of cancer

Despite extensive cell culture experiments demonstrating the transforming properties of overexpressed ERBB family receptors and ligands, and data on their amplification or overexpression in many human tumors, there exists a dearth of mouse cancer models pertaining to the ERBB family. The exception of course is the classic model of mammary tumorigenesis using the *MMTV-ERBB2* transgene (Dankort and Muller, 2000; Muller et al., 1988). Overexpression of the wild-type receptor is thought to be the primary mechanism through which ERBB2 initiates tumorigenesis in humans, but this model uses a point mutant activated version of ERBB2, a version that has never been found in human tumors. While tumors developed in *MMTV-ERBB2-WT* mice, they did so with a long latency and were found to have acquired a point mutation in the juxtamembrane domain that increased the dimerization of the receptor (Guy et al., 1992; Siegel et al., 1994; Siegel and Muller, 1996). A recent model used the activated Erbb2 cDNA under the control of the endogenous ErbB2 promoter in a Cre-regulated manner. Somatic activation of the receptor led to focal mammary tumors arising with an extended latency, and once again the modified *ErbB2* locus had undergone gene amplification leading to an increase in ErbB2 levels (Andrechek et al., 2004). Using microinjection of a Cre-expressing plasmid into embryos it was found that mice with embryonic activation of the mutant receptor surprisingly failed to develop mammary tumors. Potential explanations for these results are either (i) that activation of the allele in the germ line ablates cells that are tumor-initiating cells, or (ii) that activation in the germline leads to a rewiring of signaling pathways such that cells adapt to the constitutive oncogenic signaling. The only evidence in support of the latter comes from work on RSV where *in ovo* infection of chickens

suppressed sarcoma formation, while infection of newborn chicks led to sarcoma formation (Dolberg and Bissell, 1984; Howlett et al., 1988).

The overexpression of the ERBB ligand, TGF-α, driven by either the K1 or K14 promoter led to skin papilloma formation, primarily at sites of wounding or irritation (Dominey et al., 1993; Vassar and Fuchs, 1991), while transgenics expressing the TGF-α cDNA under the control of the metallothionein promoter developed mammary adenocarcinoma (Sandgren et al., 1990). A role for endogenous EGFR signaling in many mouse tumor types has been shown through the interbreeding of a *waved-2 (wa2)* allele, which is a hypomorphic allele with five to ten fold reduced kinase activity as a result of a V743G mutation, into transgenic backgrounds (Luetteke et al., 1994). In the case of an activated *SOS* transgenic driven by K14, this led to a reduction in the incidence and size of skin papillomas due to Egfr mediated Akt activation of survival signaling (Sibilia et al., 2000), or a 90% decrease in polyp number in the *Apc(Min)* model of intestinal tumorigenesis (Roberts et al., 2002). The recent development of conditional alleles for all *ErbB* receptors now allows the effect of complete null alleles to be examined rather than relying on hypomorphs. Deletion of *ErbB3* in the intestine of *Apc(Min)* mice decreases Akt signaling and leads to an almost complete suppression of polyp formation (Lee et al., 2009). All of this data supports a role for ErbB signaling either in tumor initiation or progression, but considering the therapeutic potential of targeting these receptors and the vast efforts aimed at developing such drugs it is surprising that up until a few years ago there were no mouse models of cancer driven by EGFR.

EGFR-driven mouse models of cancer

EGFRvIII-driven models of glioblastoma have only recently been developed and even though they rely on transgenic expression of the mutant receptor (Zhu et al., 2009) tumors only develop if *Ink4a/Arf* and/or *Pten* are deleted. In light of recent data on the heterogeneity of

EGFRvIII human tumors, combined with the ability of the mutant to transform cells in culture (Inda et al., 2010), the development of an allele where the deletion is engineered at the endogenous locus is clearly warranted. *EGFRvIII* is almost exclusively found in GBM, but recently a number of cases of squamous cell carcinoma (SCC), a subset of NSCLC, were found to contain the mutation, with an incidence of 5% (Ji et al., 2006b). A doxycycline-inducible *EGFRvIII* allele was generated and coupled to *CCSP-rtTA* to express the transgene specifically in the lung epithelium. Lung adenocarcinoma developed robustly in this model and the tumors were found to be *EGFRvIII* dependent; as a consequence the response of lung cancer patients harboring these mutations to TKIs should be assessed. An unexplained observation is that in tumors expressing EGFRvIII the endogenous Egfr protein is almost undetectable. The authors speculate about whether there exists some level of feedback due to the constitutive activity of the mutant receptor and whether such feedback may modulate levels of either the mutant or wild-type receptor, or indeed both.

Two groups have independently engineered doxycycline-inducible transgenic alleles of the kinase domain mutations that occur in NSCLC, two versions of the deletion mutant (delL747-P753insS, delL747-S752) and L858R (Ji et al., 2006a; Politi et al., 2006). In conjunction with the lung specific *CCSP-rtTA* expression of the mutant alleles leads to rapid induction of multi-focal lung tumors. This confirms a causal role for *EGFR* mutations, at least in the context of this expression level, in the initiation of lung tumorigenesis. These tumors show activated p-Akt, p-Stat3, and p-Erk, and are sensitive to mutant EGFR depletion, either through doxycycline withdrawal or via administration of TKIs. Subtle differences were encountered between the time to tumor formation between the *DEL* and *L858R* strain. While it is possible this simply reflects the integration site or expression level of the transgene, it may also recapitulate data from human patients where DEL patients treated with TKIs respond better and live longer while in an untreated cohort the L858R patients were found to have a better overall survival

(Riely et al., 2006). A careful comparison of both models will be useful to elicit the molecular basis for such differences.

A mutation in *Egfr*, L861Q, was identified in an ENU-mutagenesis screen for dominant mutations that elicited a pigmentation phenotype (Fitch et al., 2003). This strain, known as *Dsk5*, had a wavy coat, hyperpigmented footpads and hyperkeratosis in both the heterozygous and homozygous setting; these phenotypes were alleviated through interbreeding with the *wa2* allele, confirming that *Dsk5* is a hypermorphic allele. Further analysis of tissues from these mice revealed decreased protein levels of the mutant receptor, but an increase in the ratio of phosphorylated receptor to total upon administration of EGF. This mutation was later found to be a rare mutation found in NSCLC patients that respond to TKIs (Lynch et al., 2004).

Negative regulation of oncogenes

Activation of receptor tyrosine kinases in normal cells is dependent on the presence of ligands in the extracellular environment, and typically repeated stimulation with ligand is required to maintain signaling. The downstream signaling pathways that are engaged can lead to fate changes, irreversible cell decisions, or indeed transformation, and as such cells need to respond appropriately, both qualitatively and quantitatively, to such stimuli. As RTK signaling networks have evolved, negative feedback regulators have co-evolved to terminate signaling and limit the amplitude and duration of such signaling in a carefully controlled manner; an important role especially given the seeming ease with which aberrant signaling through the pathway can lead to transformation. An increased appreciation of the roles of these feedback pathways in regulating normal RTK signaling as well as their potential involvement in oncogenic signaling is emerging, and these regulators may represent novel therapeutic agents that could be co-opted to suppress signaling in the context of a tumor. Feedback regulators can be segregated into two groups based on their response time. The first group, which includes CBL and some phosphatases, are poised for action in the cell, require no protein synthesis, are

usually activated by post-translational modifications, and generally only affect acute signaling. The second group is transcribed upon receptor activation and usually acts in the timeframe following maximal activation of the receptor and serve to fine-tune or terminate the response to a pulse of signal. The following section will focus mainly on negative regulatory proteins known to function directly at the level of the receptor and not the myriad of proteins that feedback further downstream in the EGFR signaling network.

Initial thoughts as to a role for negative feedback in constraining oncogenic signaling came from studies involving ERBB2. While *ERBB2* was found to be genomically amplified in many breast and ovarian cancers there were also cases with high level expression of ERBB2 that did not have any associated amplification (Slamon et al., 1987). This suggested that a post-transcriptional means of regulating ERBB2 might be disabled in these tumors. The development of transgenic mice expressing an activated form of ERBB2 under the control of the endogenous promoter found that tumors developed with a long latency and genomic amplification always occurred (Andrechek et al., 2004). Activation and amplification may be required for tumor formation, but a potential explanation as to the underlying molecular requirement was revealed through analysis of some of the original *MMTV-ERBB2* transgenic mice. The mammary tumors that developed after a long latency were found to have acquired mutations in the juxtamembrane domain that led to constitutive activity. Subsequent analysis comparing the surrounding normal epithelium with the tumor found that while ERBB2 mRNA was overexpressed in both cases, ERBB2 protein was overexpressed only in the tumor, and ERBB3 overexpression was also found to occur only in the tumor (Carraway, 2010). One possible feedback mechanism proposed for the ERBB2 tumors is decreased expression of the ERBB3 targeting E3 ligase, NRDP1, in these tumors. Stimulation of ERBB3 leads to activation of p-Akt resulting in USP8 phosphorylation and stabilization; USP8 is required for NRDP1 stability and any interference with this feedback would result in increased receptor levels (Cao et al., 2007; Yen et al., 2006). This, together with the long tumor latency, suggests that cells adapt to

increased ERBB2 levels by initiating post-transcriptional feedback mechanisms to suppress expression of the receptor, and suggest that tumor initiation and progression may require these control mechanisms to be disabled.

Negative regulators of EGFR

CBL

CBL is perhaps the most well known negative regulator of EGFR signaling and the *C.elegans* and *D.melanogaster* homologs were uncovered in screens for suppressors of EGFR activity (Rubin et al., 2005). Such screens also identified other negative regulators, Argos, Kekkon-1, and Lrig1. CBL is the canonical acute negative regulator of EGFR activity, and following phosphotyrosine mediated recruitment to the receptor mediates receptor endocytosis, sorting, and degradation (Schmidt and Dikic, 2005). CBL leads to both mono- and poly-ubiquitination of EGFR, and while the debate as to the exact role of ubiquitination in receptor internalization continues, there is some consensus in the field that monoubiquitination results in targeting to multi-vesicular bodies and the lysosome, while polyubiquitination may lead to proteasomal degradation (Haglund et al., 2003; Huang et al., 2007; Mosesson et al., 2003).

Argos, Kekkon and LRIG-1

Tight spatial and temporal control of EGFR signaling is required during development of the drosophila egg and embryo. Two major regulators of this signaling are Argos and Kekkon-1, which happen to be the first identified negative regulators of EGFR (Ghiglione et al., 1999; Schweitzer et al., 1995). Both are transcriptionally induced by EGFR and so are classified as delayed feedback rather than members of the acute or poised, pre-existing feedback group. Argos does not interact directly with the receptor but instead has been shown to inhibit dimerization through sequestration of activating ligands such as Spitz and Gurken (Klein et al., 2008); however, the relevance to mammalian ERBB signaling is currently unknown as no homolog has been found. Kekkon-1 is a single-pass transmembrane protein whose extracellular

leucine rich repeat (LRR) domains are essential for binding the receptor and inhibiting ligand binding and kinase activity (Ghiglione et al., 2003). Kekkon-1 was found to inhibit mammalian EGFR and ERBB2 signaling, and LRIG-1 is postulated to be the mammalian homolog. The extracellular domain of LRIG-1 is similar to Kekkon-1, with 15 LRRs and 3 Ig domains, but they diverge substantially in the intracellular C-terminus. *LRIG-1* null mice develop skin hyperplasia and a 'psoriasis'-like condition, reminiscent of skin defects in mice with attenuated ErbB receptor/ligand signaling (Suzuki et al., 2002). *LRIG-1* is transcriptionally induced by EGF and interacts with all ERBB family members. Unlike Kekkon-1 whose effects are principally mediated by occluding ligand binding, LRIG-1 acts by recruiting c-CBL, leading to increased receptor ubiquitylation and subsequent degradation (Gur et al., 2004). Overexpression or hairpin suppression of LRIG-1 has been shown to modulate EGFRvIII levels, in a negative or positive direction respectively (Stutz et al., 2008), and further analysis of LRIG-1 status in human EGFRvIII gliomas, along with modulation of LRIG-1 levels in murine tumors, will shed light on the role, if any, of this negative regulator in controlling tumor initiation and progression. LRIG-1 represents one of the few examples of a protein that may play a significant role in suppression of tumorigenesis through E3 ligase recruitment to an oncogene.

SOCS proteins

Suppressor of cytokine signaling (SOCS) proteins are normally linked to regulation of cytokine receptors and indeed recent data supports a direct role for Socs3 in regulating levels of the oncogenic kinase $Jak2^{V617F}$ (Haan et al., 2009). The presence of the Socs family in C.elegans but the absence of the classical target Jak, suggested that this family may target other TKs. Socs proteins have since been found to interact with, and regulate levels of, FAK, IGFR, and c-kit (Kario et al., 2005; Zadjali et al., 2011). Initial exploration of possible links to EGFR came from studies that found Socs1 and Socs3 could bind the receptor and inhibit Stat3 signaling and that in Drosophila, the Socs5 homolog, Socs36E, when overexpressed resulted in

phenotypes similar to DER pathway loss of function alleles (Callus and Mathey-Prevot, 2002; Xia et al., 2002). In Ba/F3 cells ectopically expressing Socs5, it was shown to directly bind EGFR and to induce degradation in a ligand-dependent manner. Similar to many other negative regulators, Socs5 was shown to bind the receptor in the absence of ligand but to induce proteasomal mediated degradation only upon ligand stimulation (Nicholson et al., 2005). Using a combination of HeLA and CHO cells, Socs4 and Socs5, were transcriptionally induced upon EGF stimulation, to bind and induce degradation of the receptor in both a ligand and Cbl-independent fashion (Kario et al., 2005). The SOCS box of Socs5 was shown to be critical for this degradation to occur and suggests that this is the domain that might directly interact with EGFR. The status of the various Socs proteins, but particularly Socs4 and Socs5, in tumors that overexpress EGFR, or that express EGFRvIII or kinase domain mutations remains to be investigated.

MIG-6

Mitogen-inducible gene 6 (MIG-6), also known as ERBB receptor feedback inhibitor 1 (Errfi1), or receptor associated late transducer (RALT), was first identified in a cDNA library screen for transcripts induced in WI-38 fibroblasts upon serum stimulation (Wick et al., 1995). It was subsequently also pulled out of a two-hybrid screen in yeast using the EGFR kinase domain as bait, and EGF treatment led to recruitment of MIG-6 to the receptor in a kinase-dependent manner, while overexpression led to increased internalization of the receptor and decreased downstream signaling (Hackel et al., 2001). Increased expression of Mig-6 in EGFR expressing Rat1 fibroblasts abrogated the transforming ability of EGFR, demonstrating the potential role of Mig-6 in ERBB-driven oncogenesis. The first mouse model of Mig-6 was a K14-*Mig-6* transgenic (Ballaro et al., 2005). In a phenotype reminiscent of the *wa2 Egfr* hypomorphic allele, these mice developed a wavy coat, curly whiskers and open eyes at birth, all suggestive of decreased Egfr signaling. An examination of EGF-induced signaling in primary keratinocytes

isolated from these mice confirmed a reduction in receptor activation and downstream signaling. MIG-6 expression has been shown to inversely correlate with *ERBB2* amplification in a panel of breast cancer cell lines (Anastasi et al., 2005), with re-expression of the protein inhibiting ERBB2 signaling. A *Mig-6* knockout mouse was generated and found to be embryonic lethal on certain strain backgrounds, with up to 50% of homozygous null mice dying by 3 weeks of age (Ferby et al., 2006; Jin et al., 2007; Zhang et al., 2005). Surviving knockout mice displayed hyperproliferation and aberrant differentiation of keratinocytes, increased phospho-Egfr and p-Mapk in the skin, all of which were alleviated by crossing onto the *wa2* background or by treatment with gefitinib. Treatment of the skin of these mice with DMBA/TPA carcinogens resulted in accelerated papilloma and melanoma formation, with the former regressing 50% upon gefitinib treatment. The null mice also developed other neoplastic lesions, including lung adenomas, but due to the development of a degenerative joint disease could not be aged past 5-7 months. This was the first direct *in vivo* evidence of how disruption of a negative regulator of a proto-oncogene could play a role in tumor formation. A conditional null allele has since been generated and when deleted in the germline recapitulated the straight knockout phenotype (Jin et al., 2007). However, when *Mig-6* was deleted in the postnatal lung no adenomas developed, and this along with the abnormal lung development, including hyperplasia of alveolar type II cells and vascular defects, of germline knockout animals confirmed that the adenomas that developed in the knockout are probably a consequence of developmental defects rather than being indicative of a role for Mig-6 in suppressing Egfr signaling in the adult lung (Jin et al., 2009).

To elucidate the mechanism whereby Mig-6 binding to the receptor inhibits signaling, a series of Mig-6 fragments were crystallized with the kinase domain of EGFR (Zhang et al., 2007). Mig-6 was found to take a two-pronged approach to inhibiting EGFR signaling. A 25 amino acid portion of Mig-6 binds to the C-lobe portion of EGFR and due to the asymmetric nature of the EGFR dimer this inhibits donor-mediated acceptor activation. A fragment C-

terminal to this is also thought to bind close to the activation loop thus inhibiting kinase activity independent of dimer formation. A recent investigation into the genomic abnormalities in GBMs found recurrent loss of 1p36, with Mig-6 confirmed as the target gene (Ying et al., 2010). Mig-6 is downregulated in 50% of GBM tumor and cell line samples, and overexpression inhibits proliferation and growth in soft agar in these lines. Ectopic Mig-6 expression was found to attenuate EGFR phosphorylation and downstream signaling in these GBM lines and induced receptor downregulation through increased trafficking through the late endosome/lysosome pathway in a process that required the endosomal protein syntaxin 8 (STX8). This supports a novel role for Mig-6 in modulating EGFR activity through differential localization of the receptor. In contrast to its activity against the wild-type receptor, Mig-6 was found to not inhibit signaling from or decrease the half-life of EGFRvIII, despite retaining the ability to bind to it independent of EGF stimulation. This suggests that the primary consequence of loss of Mig-6 expression may be to release wild-type EGFR from this inhibitory interaction such that it is restored to a signaling competent state. Phosphoproteomic analysis of human bronchial epithelial cells (HBECs) stably expressing K-RasG12V, DelEGFR, EGFR-L858R or wild-type EGFR, found Mig-6 to be preferentially phosphorylated at Y394 in the mutant EGFR lines under resting conditions (Guha et al., 2008). The significance of this phosphorylation and how it affects Mig-6 function in relation to EGFR remain to be uncovered.

Mig-6 remains the most well characterized delayed feedback inhibitor of EGFR. It is usually induced 60-120 mins post EGF stimulation and as such is not classified as a delayed early gene (Amit et al., 2007a). This suggests that transcriptional induction of Mig-6 may be a contingency mechanism to ensure a period of refractoriness until cells can be restimulated with ligand, as under normal conditions activated EGFR should be internalized and degraded within this period. Elucidating the role of Mig-6 under steady-state conditions is essential as that may be a better reflection of its role in tumor cells compared to the response to high level stimulation.

SPROUTY2

SPROUTY2 (SPRY2) is a canonical feedback regulator of the MAPK pathway along with other members of the extended SPROUTY/SPRED family. SPRY2 was first identified in a Drosophila screen for modifiers of FGF induced signaling during development (Chambers et al., 2000). SPRY2 can inhibit multiple levels of the MAPK signaling cascade and is transcriptionally induced by the pathway. It can sequester GRB2, or bind and inactivate RAF kinase activity, with the exception of B-RAF, thus inhibiting downstream signaling (Rubin et al., 2005). In the context of EGFR, SPRY2 potentiates signaling via two mechanisms, both of which contribute to modulating the duration of EGFR signaling. Upon stimulation with EGF SPRY2 becomes phosphorylated at Y55, and this domain can act as a docking site for CBL. In this way SPRY2 competes with EGFR for CBL, and can also form a complex with CIN85 that is required for CBL-mediated EGFR endocytosis and clustering (Haglund et al., 2005). SPRY2 can bind hepatocyte growth factor regulated tyrosine kinase substrate (HRS) and sequester it from TSG101, an interaction required to shuttle the internalized receptor into multi-vesicular bodies and then on to the lysosome for degradation (Kim et al., 2007). Even though most studies have found SPRY2 to potentiate EGFR signaling, due to the multiple levels and directions at which it can regulate EGFR pathway signaling it is included in this section on negative regulators of EGFR.

General feedback mechanisms in tumors

The primary focus of the cancer biology field has been on tumor-promoting lesions or forward signaling pathways. There has been a growing interest in the feedback mechanisms that regulate signaling, with the possibility that they may be disabled or suppressed in tumors. Recently a new concept has emerged in the targeted therapy field; namely, that treatment with inhibitors may indirectly lead to reactivation of suppressed signaling pathways through removal of active negative feedback mechanisms. This may allow tumors to survive by co-opting these pathways and minimizes the therapeutic effect of the inhibitor. Whether disabling negative

feedback mechanisms is sufficient to initiate tumorigenesis or only plays a role in tumor progression by relieving feedback on oncogenic signaling remains to be delineated.

In an era before the details of the EGFR signaling pathway had been established it was noticed that cycloheximide (CHX) treatment of NIH3T3 cells prior to growth factor stimulation led to enhanced production of c-fos mRNA, one of the first pieces of evidence suggesting a role for induction of feedback inhibitors as a means to terminate signaling (Greenberg et al., 1986). To gain further insight into how transcriptionally-induced feedback can potentially modulate the signaling induced by growth factors and result in a robust response, HeLa and MCF10A cells were stimulated with EGF and the response analyzed both at the gene expression and phospho-protein level (Amit et al., 2007a). Inhibition of translation using CHX revealed that *de novo* protein synthesis was necessary to attenuate ERK signaling post-EGF treatment, confirming a role for transcription in feedback inhibition of the pathway. At a series of time intervals after stimulation, waves of gene expression are induced, and the genes in each set can be classified as immediate-early genes (IEGs), such as the transcription factors FOS and EGR1, or delayed early genes (DEGs), such as FOSL1 and NAB2, which are induced by IEGs. The major role of DEGs is to inhibit the transcription of the IEGs that induced them or to degrade these mRNAs, as occurs with ZFP36, which targets mRNAs with AU-rich elements (AREs), a feature of IEGs. This feedback combination is responsible for the waves of gene expression produced. Analysis of gene expression data sets from a panel of tumor types found DEGs to be downregulated in ovarian, lung and prostate cancer. This points to a conserved mechanism for attenuation of growth factor signaling that seems to be independent of stimulus and cell type. Subsequent analysis of the same system found that a collection of 23 miRNAs were downregulated at the onset of stimulation, and their targets were predominantly IEGs, thus facilitating a rapid induction of IEGs (Avraham et al., 2010). miRNAs are thought to act predominantly as fine-tuners of gene expression and their role in defining features of EGFR signaling such as the amplitude and duration of response remain to be characterized.

The MAPK pathway is a well-studied example of a pathway where the output is regulated by a series of interconnected positive and negative feedback loops. Despite high levels of p-ERK in a spectrum of tumors with mutations in the MAPK pathway, ranging from EGFR/ERBB2 mutation or overexpression, to *RAS* mutation, or *B-RAF* mutation, only cells expressing the latter are sensitive to MEK inhibition (Solit et al., 2006). To uncover the molecular basis for this difference in sensitivity and why p-ERK levels are not a good predictor of signaling flux through the pathway, a panel of B-RAF^{V600E} cell lines were treated with the MEK inhibitor, PD0325901, for eight hours and gene expression profiling was carried out to determine the set of genes whose expression was altered upon acute MEK inhibition (Pratilas et al., 2009). A gene-set of 52 was identified, with 48 genes undergoing downregulation upon MEK inhibition. These could be classified as either transcription factors that are known ERK targets, ETV1/4/5, FOS, EGR1, or as feedback regulators of the ERK pathway, DUSP4/6, SPRY2/4. The surprising result came when the same inhibitor experiment was carried out in non-B-RAF mutant cells that displayed high p-ERK; there were no significant changes in gene expression upon treatment with the MEK inhibitor. p-MEK levels were then examined and found to segregate with B-RAF^{V600E} and sensitivity to the inhibitor. Paradoxically, upon treatment of the non-BRAF mutant cells with a MEK inhibitor the levels of p-MEK actually increased over time. The model proposed to explain this differential sensitivity is that in RTK-activated tumors any increase in p-ERK is countered by an equivalent level of feedback both at the level of ERK using DUSPs, and at the level of RAF, leading to a steady state situation where p-ERK levels are balanced by low levels of p-MEK and low levels of feedback, resulting in negligible ERK pathway transcriptional output. B-RAF^{V600E} leads to high p-MEK and p-ERK and while the latter is dampened due to the associated high levels of transcriptionally induced feedback including DUSPs, B-RAF and MEK are somehow insensitive to feedback and continue to generate signaling through the MEK/ERK pathway. This reinforces the idea that the readout used to assess flux through the MAPK pathway can dramatically affect the assessment of potential

inhibitor sensitivity. This seminal paper also bolstered the concept that different oncogenic lesions may be more or less susceptible to particular types of feedback, and that such feedback may need to be disabled to unmask the full potential of an oncogenic lesion. Not surprisingly the cell has evolved numerous ways to keep signaling through growth factor related pathways under control, even in the context of tumors.

In a mouse model of K-Ras^{G12D} NSCLC the Erk pathway is not detectably activated in early lesions (Jackson et al., 2001). An examination of MEFs carrying this allele also found that expression of this oncogenic protein led to reduced basal levels of p-Erk and p-Akt, and diminished p-Mek and p-Akt upon restimulation of serum-starved cells, both in maximal intensity and duration of signaling (Tuveson et al., 2004). Recent data has found a correlation between advanced lesions, K-Ras amplification, loss of feedback inhibitors such as Spry2, and an increase in p-Erk signaling (Feldser et al., 2010; Junttila et al., 2010). This is a classic example of how in the course of natural tumor development cells attempt to keep oncogenic signaling in check using normal feedback mechanisms, and that tumor progression is associated with these pathways being either disabled or overwhelmed by forward signaling.

Due to the frequency of PI3K pathway mutations in breast cancer (approximately 70%) and the associated downstream activation of mTOR, rapamycin analogs have been examined as a potential therapeutic avenue (Lopez-Knowles et al., 2010). Despite mTOR inhibition, minimal therapeutic benefits resulted from rapamycin administration. This was related to the concurrent disabling of the S6K negative feedback loop on IRS1-PI3K, leading to either increased AKT (Cloughesy et al., 2008; O'Reilly et al., 2006; Tabernero et al., 2008) or increased MAPK signaling in many cases (Carracedo et al., 2008). A strategy to counteract this using a combination of PI3K/mTOR inhibitors was recently tested. Unexpectedly, ERK phosphorylation was increased in ERBB2 overexpressing breast cancer cell lines treated with the dual inhibitor. This was due to AKT inhibition resulting in increased FOXO3a in the nucleus, increased transcription of ERBB2/3, and these activated heterodimers then engaging

downstream signaling pathways such as MAPK (Serra et al., 2011). A similar study on a panel of cell lines, not all of which overexpress ERBB2, found that treatment with PI3K-AKT inhibitors leads to an induction of ERBB3, IGF-1R, EphA7 and IR, through a similar mechanism of mTOR inhibition relieving IRS-1 phosphorylation and AKT inhibition relieving inhibitory FOXO phosphorylation (Chandarlapaty et al., 2011). Both studies found that a combination of AKT and ERBB inhibitors could inhibit proliferation. This work highlights the complexity of the wiring of signaling pathways in tumor cells, and exemplifies the perhaps surprising notion that while oncogenes may activate certain pathways, others may be actively silenced through negative feedback mechanisms. The potential release of different signaling pathways from suppressive signaling upon inhibitor administration further complicates the notion of combination therapy. The possibility exists that this modulation of the wiring of signaling pathways by oncogenes, with certain pathways activated at the expense of others, contributes to oncogene addiction by funneling signaling into one or a few pathways, and a greater understanding of the underlying networks will profoundly impact therapeutic strategies.

Gene Amplification

Original description

Gene amplification was originally described in developmental processes, including rDNA amplification in *Xenopus laevis* oocytes, the amplification of chorion genes in ovarian follicle cells allowing for rapid eggshell formation in *Drosophila*, and the classic example of rDNA amplification in *Tetrahymena* (Brown and Dawid, 1968; Spradling, 1981; Yao et al., 1979). In all cases the end result is an increase in gene dosage usually accompanied by a parallel increase in mRNA/protein. Genetic instability is one of the original hallmarks of cancer (Hanahan and Weinberg, 2000), and the recent development of tools such as dense SNP arrays and deep sequencing has allowed genomic alterations to be assessed with increasing resolution and on a

genome-wide scale (Beroukhim et al., 2010; Weir et al., 2007). This has revealed a large number of genes that are amplified across many tumor types, either through gain of whole chromosomal arms or through focal amplification, the consequence of most of which remain to be assessed.

Mechanism

Oncogene amplification is not thought to occur via the same aberrant origin firing as occurs with amplification of rRNA and the chorion genes. Instead, one of the principal mechanisms by which gene amplification in tumors is posited to occur is through the breakage-fusion-bridge (BFB) cycle, proposed by Barbara McClintock in the 1940s to explain genomic instability in maize (McClintock, 1941). The model is as follows: under normal conditions if a double strand break (DSB) occurs cells will arrest and either repair the lesion or undergo apoptosis. When such a break occurs in tumor cells they often will continue through the cell cycle with the unrepaired break due to the absence of the appropriate checkpoints. Upon entering S-phase the broken chromosome now undergoes replication and the availability of uncapped ends allow sister chromatid fusion to occur. This leads to an anaphase bridge forming due to the presence of a dicentric chromosome, and the ensuing force generated as the chromosomes attempt to segregate results in a break, and if the break occurs telomeric to the gene of interest then a duplication has formed. Unless telomeres are added to cap the broken chromosome, or it undergoes fusion with another chromosome, the cycle repeats, leading to high-level amplification of the gene.

Gene amplification and cancer

Gene amplification of *MYCN* and *ERRB2* was initially observed in neuroblastoma and breast cancer respectively, using a combination of southern blotting and fluorescence in situ hybridization (FISH) (King et al., 1985; Schwab et al., 1983), and a correlation later emerged between amplification and survival or time to relapse (Brodeur et al., 1984; Mass et al., 2005;

Slamon et al., 1987). Amplification of oncogenes is typically analyzed at the end-stage of the process as there currently exists no reliable way to instigate such a process in a targeted manner in tumor cells. As such the exact path(s) by which amplicons evolve can only be surmised, but substantial evidence exists to support the role of the BFB model in their development. The BFB model for amplification makes the following predictions about the composition of tumor amplicons: (i) the amplified regions should be arranged as inverted repeats, (ii) the amplicons should be arranged in a so-called 'ladder pattern', with any given point in a repeat unit a set distance from its counterpart in the preceding repeat unit, (iii) the breakpoints that define the amplicons should be somewhat uniform, (iv) the telomeric portion of the chromosome should be missing or replaced with other sequences, and (v) anaphase bridges may be visible. Amplified oncogenes do not always satisfy all of these criteria, which may be a consequence of further processing of these amplicons over time, but evidence for all of these predictions of the model have been found (Gisselsson et al., 2000; Hellman et al., 2002). Other possible mechanisms, such as unequal sister chromatid exchange, or looping out of amplicons to form extrachromosomal elements known as double minutes, could also combine with BFB cycles to lead to gene amplification by some hybrid mechanism.

The mechanisms underlying gene amplification have been extensively studied using a cell culture model of drug-induced amplification of target loci. It is not known if the underlying mechanism mimics completely that in tumor cells but there are no doubt overlapping features. Amplification of the *CAD* or *DHFR* gene can be induced by treatment of cells in culture with the cytotoxic drugs PALA and methotrexate respectively. PALA-induced amplification was found to be dependent on the absence of p53, as amplification failed to occur in fibroblasts from Li-Fraumeni patients that had lost the remaining wild-type p53 allele when wild-type p53 was added back to these cells, and instead these cells arrested in the presence of drug (Livingstone et al., 1992; Yin et al., 1992). This suggested that without the p53 DNA damage checkpoint cells with DNA breaks were aberrantly progressing through the cell cycle, rather than arresting, and

possibly undergoing a breakage-fusion-bridge (BFB) cycle as a result. These studies were also some of the first to make the connection between forms of DNA damage, aberrant repair and tumor promoting events. Initial evidence that DNA breaks were the initiating event of gene amplification was found in mammalian cells (Windle et al., 1991), and the characterization of fragile sites, genomic locations that are hypersensitive to replication-stalling drugs and prone to breakage, led to the idea that breaks at these sites might be the major initiating event. Fragile sites are scattered throughout the human genome, numbering around 100, and have been shown to be late-replicating, and undergo a degree of chromatin reorganization prior to breakage (Hellman et al., 2002). The initial connection between fragile sites and cancer was made by noticing the overlap between the chromosomal position of many fragile sites and that of the small number of oncogenes known at the time, many of which were later shown to undergo amplification (Yunis and Soreng, 1984). A common fragile site, FRA7G, was recently shown to mark amplicon boundaries of *MET* in a gastric carcinoma cell line, and the amplicon displays many other features indicative of BFB cycles having occurred (Hellman et al., 2002). While fragile sites may mark the boundary of an amplicon it should be noted that they are often far from the 'target' gene, hence it is their sensitivity to breakage and instability that is important rather than a direct selection for a break at that position.

The amplification of oncogenes in human tumors, and the transforming ability of these genes when overexpressed, either in cell culture or in mouse models, supports a role for gene amplification in tumor initiation. Little is currently known about the role of gene amplification in tumor progression or metastasis and how, if at all, a graded rather than instantaneous transition to high-level expression alters tumor development. The importance of gene amplification in therapeutic sensitivity is also well documented, from the amplification of *ERBB2* (Mass et al., 2005) to *EGFR* (Cappuzzo et al., 2005; Hirsch et al., 2006), to resistance to TKIs through *BCR-ABL* amplification (Gorre et al., 2001), or *MET* amplification (Engelman et al., 2007), and a clearer understanding of the mechanisms involved is needed.

Evidence for amplification from mouse models of cancer

The use of GEMs has provided important mechanistic insight into the process of gene amplification. Telomere dysfunction would be expected to contribute to the amplification process in tumors and *Terc*$^{-/-}$ mice develop tumors with an increased frequency of amplifications than is commonly found in mouse tumors that develop on the background of longer telomeres (O'Hagan et al., 2002). Mice deficient in components of the non-homologous end joining (NHEJ) complex, such as *Lig4* or *Xrcc4*, are embryonic lethal unless combined with loss of *p53*. These compound mutant mice go on to develop lymphomas harboring amplification of a *IgH/c-myc* translocation (Zhu et al., 2002), and by crossing with a *Rag2*$^{-/-}$ mouse the initiating event for the amplification process was shown to be a Rag2 endonuclease induced DSB (Difilippantonio et al., 2002). In the context of solid tumors and cell types that don't routinely undergo DSBs, deletion of both *Xrcc4* and *p53* in neuronal progenitors led to medulloblastomas harboring amplifications of *N-myc* and *CyclinD2*, while *Lig4* heterozygosity on an *Ink4a/arf*$^{-/-}$ background led to sarcoma formation containing amplifications of the Mdm2 gene among others (Sharpless et al., 2001; Yan et al., 2006).

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Chapter 2

The level of oncogenic Egfr is a critical determinant of tumor initiation in the lung

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Keara Lane performed all experiments in the laboratory of Tyler Jacks except for the internalization assays, which were performed by M. Lazzara in the laboratory of Doug Lauffenburger.

Abstract

Oncogenic lesions in RTKs typically take the form of mutation or gene amplification, with both approaches resulting in aberrant signaling through downstream signaling pathways. The specific contribution of a mutation or the expression level of a given gene to tumor development or therapeutic response is generally unknown, but these lesions are often assumed to result in similar outcomes. The discovery of *EGFR* kinase domain mutations in NSCLC patients who responded to TKIs represented the first example of targeted therapy for lung cancer. The dependence of human tumors on sustained mutant receptor expression for survival along with the finding that ectopic expression of these mutations was transforming all suggested that these mutations are causal events and would be sufficient to induce tumor formation in the lung. To investigate this, and to further our understanding of how deregulated signaling through the mutant receptor could initiate tumor formation, we generated a conditional and constitutive knock-in allele of one such mutation, L858R, at the endogenous murine *Egfr* locus. Expression of mutant Egfr fails to induce lung tumors in these mice but an analysis of germline mutant mice found the mutant receptor to be downregulated. These data suggest that normal cells can respond to an oncogenic lesion by upregulating negative feedback pathways to counteract the induction of aberrant signaling, and disabling these feedback mechanisms may be an essential component of the progression of *EGFR* mutant tumors.

Introduction

Aberrant regulation of RTK signaling responses, such as proliferation, migration, and differentiation, is known to contribute to tumor formation. Although they account for a relatively small portion of the proteome, RTKs were among the first oncogenes cloned and over 30% of the RTK family is mutated or aberrantly expressed in human tumors. The mechanisms through which proto-oncogenes, including RTKs such as the ERBB family, are activated are generally limited to either point mutation, overexpression, or translocation, either alone or some combination thereof, the end result of which is a decoupling of the proto-oncogene signaling from its normal physiological constraints. Many human tumors have been documented to harbor oncogenic mutations, amplifications or both, but how, or if, these tumor subsets differ in regards to signaling, tumor evolution, or therapeutic response, is currently unknown.

With tremendous foresight, but based solely on the overexpression or mutation of ERBB family members in human tumors and the ability of combinations of ERBB members to transform cells in culture, targeted therapies against EGFR and ERBB2 were initiated, with the expectation that aberrant receptor signaling would constitute so called 'driver' mutations and render tumors 'oncogene-addicted' (Mendelsohn, 1990; Weinstein, 2002). Advances in sequencing and genome-wide SNP analysis has documented an array of mutations and other genomic aberrations in human tumors (Bardelli et al., 2003; Beroukhim et al., 2010; Sjoblom et al., 2006; Weir et al., 2007; Wood et al., 2007); the number of lesions in any given tumor makes their dependence on a single aberrantly expressed gene or pathway, and the corresponding response to inhibition, all the more striking (Bollag et al., 2010; Flaherty et al., 2010; Lynch et al., 2004; Paez et al., 2004).

The idea that a single point mutation in *RAS* could elicit transformation of NIH3T3 cells (Reddy et al., 1982; Shih and Weinberg, 1982; Tabin et al., 1982; Taparowsky et al., 1982), was later amended when the same cDNA failed to transform primary rat embryonic fibroblasts, unless either myc or E1A was co-expressed (Land et al., 1983; Ruley, 1983). The multi-step

model of tumorigenesis followed, with the idea that multiple layers of control need to be abrogated if tumor formation is to occur. Transgenic mice ectopically overexpressing mutant forms of RAS developed tumors, but as had previously been observed in cell culture the addition of Myc had a synergistic effect (Quaife et al., 1987; Sinn et al., 1987). Human tumors with Ras mutations are also often found to have amplification of the mutant allele (Soh et al., 2009). The question remained as to the number and type of lesions that were necessary to induce tumor formation in a given context, and the nature of the effect of a single oncogenic point on cellular homeostasis.

The advent of mouse modeling provided the opportunity to engineer sophisticated models in which the number and type of lesions could be exquisitely controlled, on both a spatial and temporal level. The phenotypic consequences of a single mutation in a proto-oncogene was finally resolved through the use of two elegant mouse models, the *K-Ras*^{G12D} 'latent' allele, in which a duplication of exon 1 at the endogenous locus is spontaneously resolved, most likely through intrachromosomal recombination, resulting in a single copy of mutant *K-Ras* (Johnson et al., 2001), and the *K-Ras*$^{LSL-G12D}$ allele, which contains a stop element in intron 1 that is removed upon Cre-mediated recombination (Jackson et al., 2001), restoring expression of the mutant allele. In both models a single-copy of activated *K-Ras* was sufficient to induce adenomas, and to some extent adenocarcinoma formation in the lung, and in combination with loss of p53 disease severity and progression could be accelerated. Overexpression of oncogenic Ras in primary cells leads to increased effector pathway signaling and oncogene-induced senescence (OIS) (Serrano et al., 1997). In contrast, single copy activation of mutant K-Ras in primary MEFs paradoxically decreased canonical effector pathway signaling and failed to engage the tumor suppressor pathways of p53 and Ink4a/Arf, resulting in partially transformed MEFs (Tuveson et al., 2004). Together with other studies on the contribution of levels of oncogene expression to transformation (Murphy et al., 2008; Sarkisian et al., 2007) this has led to the idea that point mutations in oncogenes may initiate aberrant, but

low-level signaling, which alone may result in hyper-proliferation, but only in concert with deletion of tumor suppressor genes is the potential for OIS bypassed and transformation induced. High level expression of oncogenes is therefore problematic, and in the context of otherwise wild-type cells either such signaling needs to be constrained by a parallel increase in the induction of feedback mechanisms, or overexpression is selected against and restricted to late-stage tumors when OIS can no longer be induced.

EGFR, the prototypic receptor tyrosine kinase, was originally identified to be homologous to the viral oncogene, v-erbB, which abrogates normal cellular control mechanisms resulting in transformation through the ability to signal in a ligand-independent manner and an insensitivity to CBL mediated downregulation (Peschard and Park, 2003). EGFR has been pursued as a therapeutic target, primarily as a result of its overexpression in many tumor types (Mendelsohn, 2003), along with the potential to target it with either monoclonal antibodies or kinase inhibitors. Kinase domain mutations in *EGFR* were originally discovered as part of an attempt to understand the limited, but dramatic response of NSCLC patients to the EGFR directed kinase inhibitors, gefitinib or erlotinib (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004). These mutations are found primarily in non-smokers, women, and those of Asian ethnicity, and the most common mutations accounting for 90% of all cases are either a deletion in exon 19 or a point mutation, L858R, in exon 21. Tumors, and cell lines, with EGFR kinase domain mutations are dependent on mutant EGFR signaling for survival (Sordella et al., 2004), which seems to be primarily mediated through ERBB3 activation of AKT (Engelman et al., 2005); the finding that the principal TKI resistance mechanism is a secondary mutation in *EGFR* further supports the idea that oncogenic EGFR signaling is the driving mutation in these tumors. Ectopic expression of these mutant receptors transformed NIH3T3 cells in a partially EGF-dependent manner (Greulich et al., 2005) and led to IL-3 independent growth of Ba/F3 cells, a condition associated with transformation (Jiang et al., 2005).

The predictive value of mutation or FISH positivity for determining response to TKIs has been a contentious issue for the EGFR field (Hirsch et al., 2003; Hirsch et al., 2006; Sholl et al., 2010), and while the relative impact of wild-type EGFR expression levels on response remains to be determined, 75% of patients with mutations show a response (Tsao et al., 2005). A more thorough examination of EGFR mutant tumors and cell lines has found that amplification is concurrent with mutation in approximately 70% of cases, with preferential amplification of the mutant allele (Nomura et al., 2007; Soh et al., 2009; Takano et al., 2005). The relative contribution of mutation and amplification to tumor development and therapeutic response is unknown, but data from early lesions would predict that mutation precedes amplification (Tang et al., 2005; Tang et al., 2008).

At the time of their discovery the oncogenic nature of these mutations from both a structural and mechanistic perspective was unknown, and the modes of resistance to TKIs, although predicted based on previous experience with imatinib (Gorre et al., 2001), were undocumented. We engineered the L858R mutation into the endogenous *Egfr* locus, creating both a germline and conditionally regulated form of the mutant allele, to further our understanding of how deregulated signaling through mutant EGFR can initiate tumor formation. The premise of this approach was that in contrast to both EGFR mutant human tumors and cell lines that contain other unknown modifications in the background, cells from these mice could be used to understand mutant receptor signaling in an otherwise wild-type setting, and the evolution of lung tumors carrying a mutant receptor could be tracked from incipient cell through to adenocarcinoma, and through treatment with TKIs these mice would serve as excellent model systems in which to dissect out mechanisms of resistance. Contrary to expectation, mice carrying this allele, either alone or in combination with deletion of a series of tumor suppressor alleles, failed to develop lung tumors. This result stands in contrast to the very efficient induction of tumors in an analogous system involving an oncogenic *K-Ras* allele developed in our laboratory (Jackson et al., 2001), and to transgenic models utilizing doxycycline inducible alleles

of the mutant receptor, which in combination with a lung-specific rtTA induce NSCLC-like lesions that are sensitive to gefitinib or erlotinib (Ji et al., 2006; Politi et al., 2006). Our results support a model where expression of mutant *Egfr* from the endogenous locus is insufficient to initiate tumor formation, at least in the mouse, and have led us to explore the hypothesis that a threshold expression level of oncogenic Egfr is required for tumor initiation.

Results

Generation of conditional and germline alleles of Egfr^{L858R}

To understand the role of Egfr^{L858R} in lung tumorigenesis we set out to construct a conditional allele of this mutation in the mouse; closely modeling the approach used to generate the *K-Ras$^{LSL-G12D}$* allele previously made in the lab (Jackson et al., 2001). In an effort to minimize the incorporation of exogenous sequences into the mouse genome we used recombineering technology to engineer all targeting constructs (Copeland et al., 2001). In this way we were no longer reliant on the restriction sites that were present in the genomic region to be targeted and did not have to leave any remnants of vectors in the genome as a result of cloning strategies.

Intron 1 of *Egfr* is approximately 100kb and as such we were required to take a two-step targeting approach to generate a conditional allele of *Egfr^{L858R}*. A schematic of the final alleles is shown in Figure 1A. A FRT-neo-FRT cassette was inserted into intron 20-21, and the point mutation (T->G) into exon 21. ES cell clones were screened by southern blot for the presence of the FRT-neo-FRT cassette (Figure 1B) and then we carried out a PCR reaction for exon 21 to confirm that the mutation was co-integrated into the genome. Once positive clones were identified these were re-targeted with the lox-stop-lox cassette, which was inserted into intron 1, and positive clones identified by southern blotting (Figure 1C). This second targeting event could have occurred in *cis* or in *trans* to the L858R targeting event and to determine the outcome we took advantage of the elimination of an Msc1 restriction site upon the T to G transition that accounts with the L858R mutation. In short, mRNA was isolated from doubly-targeted ES cell clones, and an RT reaction for Egfr was carried out and the product digested with Msc1. If the targeting events had occurred in *cis* then no mutant Egfr mRNA should be made and all of the product should digest with Msc1. If however, they occurred in *trans* then mutant mRNA would be made, and would be resistant to cleavage. ES cell clones in which the events had occurred in *cis* were identified (data not shown) and injected, as were clones from

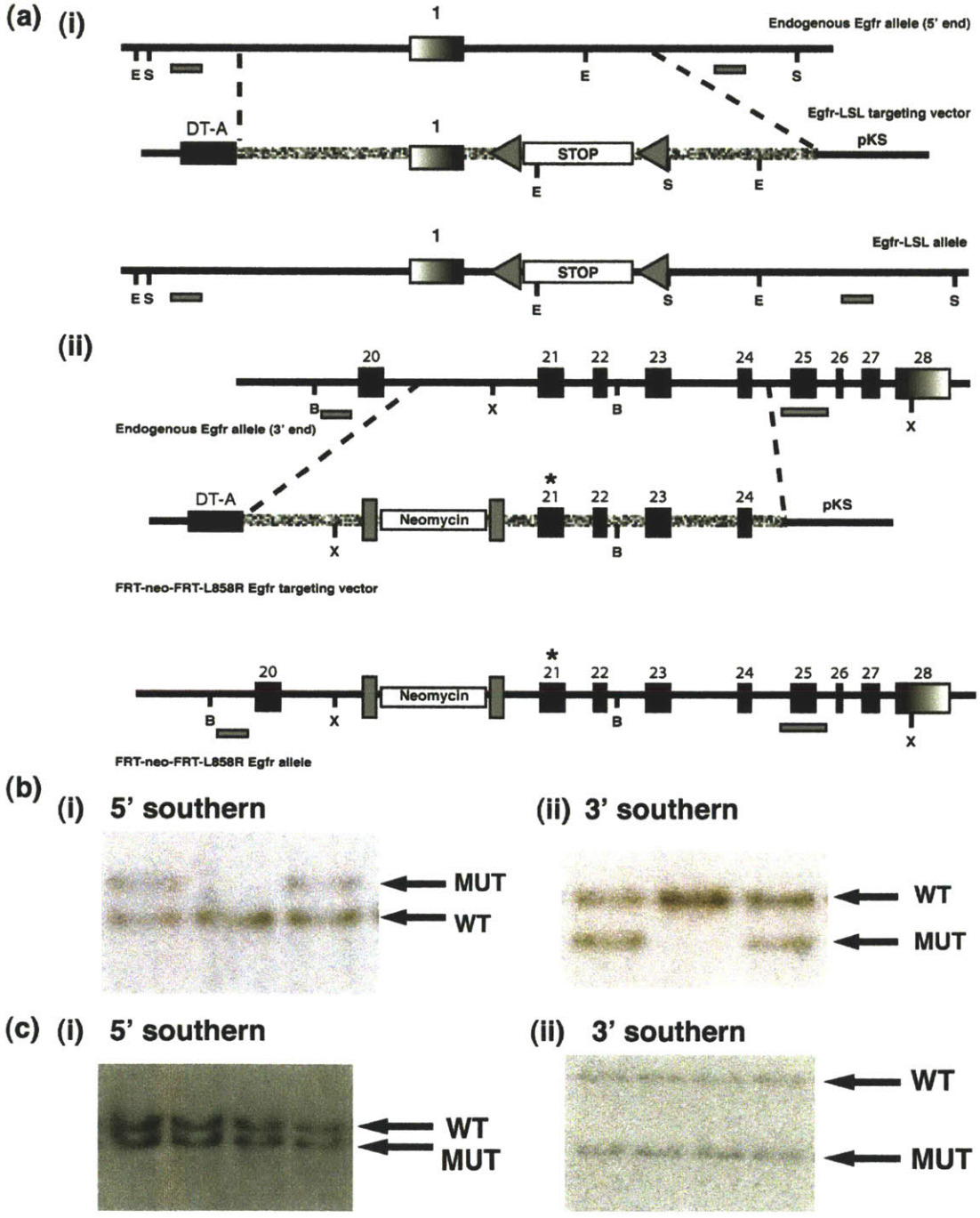


Figure 1. Generation of conditional and constitutive alleles of Egfr^{L858R}.

(a) Schematic representing both targeted alleles. **(b)** Southern blot analysis of ES clones for FRT-neo-FRT-L858R targeting was carried out using BamH1 for the 5'end and Xba for the 3' end. Note: the FRT-neo-FRT cassette was later removed by crossing the mice to ß–actin Flpe transgenics. **(c)** Southern blot analysis of ES clones for the LSL was carried out using EcoRV for the 5'end and Sac1 for the 3'end.

the first round that just had the mutation, but no LSL.

Viability of the 'germline' allele and the absence of a tumor phenotype

The first surprising result was the germline transmission of the constitutive allele, hereafter referred to as the 'germline' allele, and the subsequent viability of the hetero- and homozygous mutant mice. Almost all oncogenic mutations are embryonic lethal if expressed in the germline, especially if in a constitutive manner in every cell (Shaw et al., 2007; Tuveson et al., 2004). These germline mutant mice have the classical 'wavy' coat characteristic of many hypomorphic *Egfr* alleles, such as *wa2 (Luetteke et al., 1994)*, and also of some hypermorphic alleles such as *Dsk5 (Fitch et al., 2003)*, and they also have hyper-pigmented footpads. These mice were much leaner than their littermates but apart from that no obvious defects were noticed. We proceeded to generate MEFs from these mice and they failed to show anything but marginal signaling differences in response to mEgf stimulation, and this coincided with the absence of any features of transformation in culture, even when cultured in the presence of low doses of ERBB ligands.

Cohorts of $Egfr^{LSL\text{-}L858R/+}$ mice were infected with adenovirus Cre and aged. Surprisingly, no lung tumors developed in these mice or in the germline heterozygous or homozygous mutant mice. This was in contrast to transgenic models constructed at the same time that used a lung specific *CCSP-rtTA* to drive expression of doxycycline-inducible alleles of mutant EGFR; these mice developed multi-focal lung adenomas within 4-8 weeks of induction, and these tumors were found to be dependent on mutant EGFR signaling for their survival (Ji et al., 2006; Politi et al., 2006). We generated cohorts of mice that combined the $Egfr^{LSL\text{-}L858R}$ allele with the following conditional tumor suppressor deletion alleles: $p53^{FL/FL}$; $pten^{FL/FL}$; $Ink4a/Arf^{FL/FL}$; $Nf2^{FL/FL}$; and infected them with adenovirus-Cre. None of these mice developed lung tumors. One possibility was that perhaps adenovirus-Cre was not infecting the correct target cell in the lung; to address this we also combined the $Egfr^{LSL\text{-}L858R}$ allele with the $R26^{CreER}$ allele, which is ubiquitously

expressed, and administered tamoxifen to the mice. These mice also failed to develop lung tumors. This result along with the fact that the germline mutant mice express mutant Egfr in all cells of the lung yet fail to develop tumors led us to surmise that it was unlikely that the absence of tumors was because we were not activating Egfr in the appropriate cell type.

We confirmed that the LSL cassette was functioning appropriately by generating MEFs and blotting for mutant Egfr post-Cre administration (Figure 2A, i), confirming that the LSL could be removed and expression of the mutant receptor activated. We also confirmed that the mutant receptor could be activated by stimulation with EGF (Figure 2A, ii). The prospect of leaky expression of the mutant receptor was a concern as based on studies with ERBB2 there was the possibility that expression of the mutant receptor in the germline would lead to a rewiring of signaling such that the cells could now tolerate expression of the mutant receptor without the induction of tumors (Andrechek et al., 2004). Due to the absence of any EGF stimulated response in $Egfr^{LSL\text{-}L858R/LSL\text{-}L858R}$ MEFs that have not been exposed to Cre we concluded that there is minimal to no leaky expression of the receptor (Figure 2A, ii). Further evidence indicating the LSL was functional was that no viable $Egfr^{LSL\text{-}L858R/LSL\text{-}L858R}$ mice were ever detected, and when we tried to make MEFs of this genotype we had to harvest embryos at E11.5-E12.5; both pieces of data are in accordance with the known embryonic lethality of the Egfr knockout mouse which the LSL/LSL would be expected to phenocopy.

We wanted to confirm that the mutant receptor displayed features attributed to mutant EGFR in other systems. We chose to look at the internalization rate of the mutant receptor as the result is highly quantitative and the mutant receptor has previously been shown to internalize at a markedly lower rate than the wild-type (Hendriks et al., 2006). EGF internalization rates for a panel of wild-type, germline L858R/+, and germline L858R/L858R were determined. We found that a decreased internalization rate was indeed a property of the

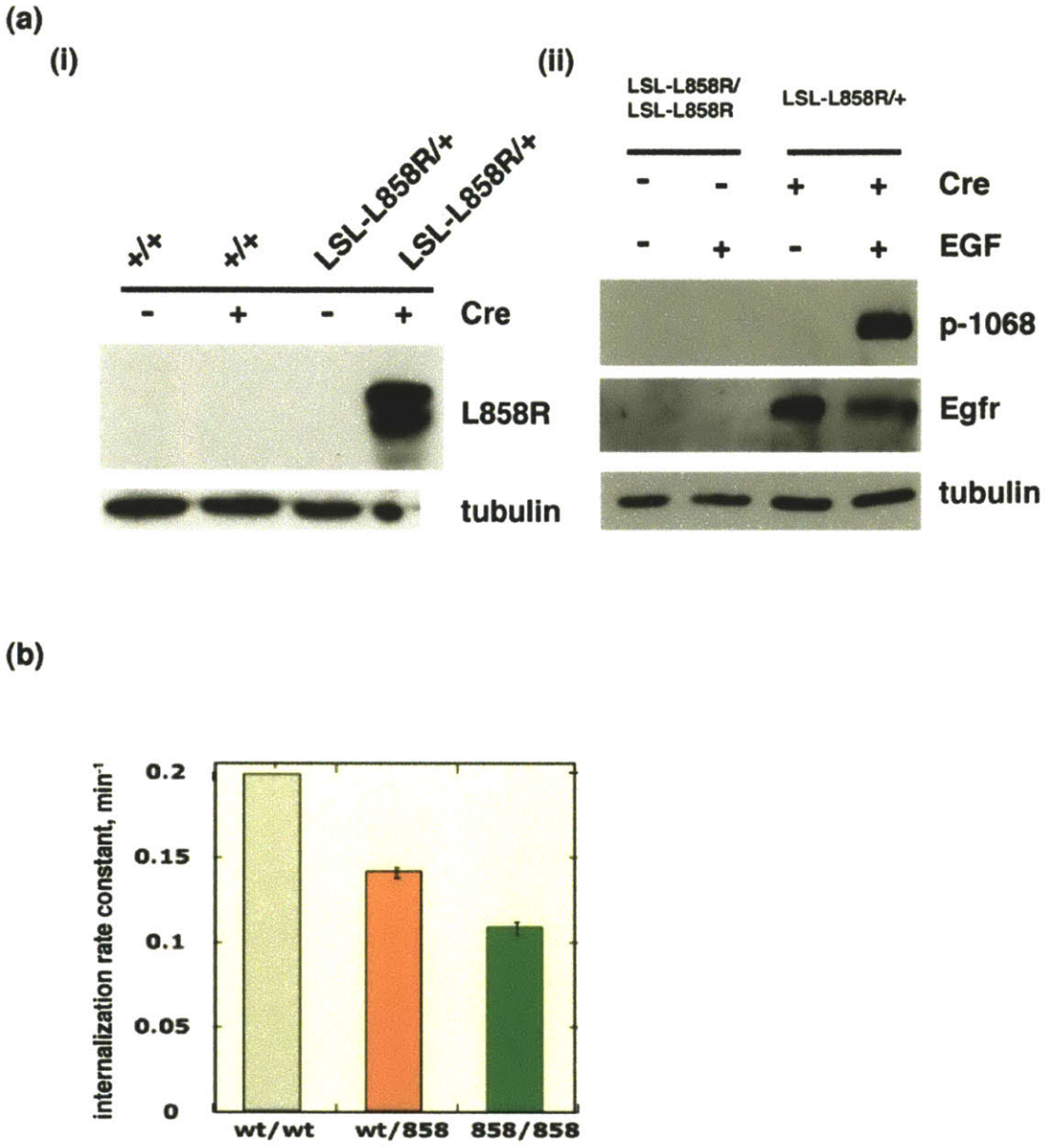


Figure 2. The LSL is functional, and mutant Egfr internalizes at a lower rate.

(a) (i) wild-type and conditional mutant Egfr MEFs were mock-treated or treated with adenovirus Cre and induction of mutant Egfr protein assessed by blotting with an antibody specific for $Egfr^{L858R}$. The $Egfr^{LSL\text{-}L858R}$ allele can be successfully recombined leading to expression of mutant protein. **(ii)** LSL-L858R/LSL-L858R fail to show total or phospho-Egfr1068 even in the presence of Egf, indicating that the LSL is functioning to silence gene expression at the locus. **(b)** The internalization rate constant in response to short-term EGF stimulation of MEFs of the indicated genotypes. Mutant Egfr clearly internalizes at a lower rate than the wild-type receptor.

mutant receptor on an otherwise isogenic background (Figure 2B) and the internalization rate appeared to track with the number of copies of the mutant receptor.

Murine Egfr^{L858R} is oncogenic when ectopically expressed in the lung

One factor that distinguished the transgenic alleles from our knock-in allele was that they used the human EGFR cDNA for their studies. The possibility existed that despite the high level of conservation that exists between human and mouse EGFR, especially in the kinase domain, that there were subtle differences that accounted for the absence of a tumor phenotype in our model. To address this we generated lentiviruses that would ectopically express either the wild-type or mutant murine receptor (Figure 3A). We infected wild-type mice with these viruses and waited. 12-18 months post-infection the mice that received the L858R virus developed tumors. We confirmed the presence of the mutant cDNA by carrying out laser-capture microscopy (LCM) on these samples (Figure 3C). We carried out limited IHC on these tumors but found that they stained positive for L858R and for total Egfr (Figure 3D). These results confirmed that mEgfr^{L858R} is oncogenic in the lung, and viral delivery is a suitable approach to target cells that are sensitive to mutant Egfr expression. These studies were carried out solely to confirm the oncogenic nature of mEgfr^{L858R} and tell us nothing about its oncogenicity relative to hEGFR^{L858R}. We repeated these studies by infecting germline L858R/L858R mice and found that they too succumbed to tumors with a similar latency to wild-type mice. This confirmed that the germline expression of the mutant receptor in cells does not rewire signaling to an extent that the cells are no longer sensitive to the presence of an increased level of Egfr at some later stage. Data from human NSCLC cell lines and patients have suggested that ErbB3 expression is a critical component of mutant Egfr signaling, and that ErbB2 may also modulate therapeutic response to TKIs. Now that we could express cDNAs *in vivo* we wanted to address whether expression of ErbB2 or ErbB3 was a limiting factor for transformation of the lung epithelium. We generated lentiviruses expressing these cDNAs driven either by pGK or CMV promoters and infected wild-type and L858R/L858R germline mutant mice. While no tumors developed using this approach

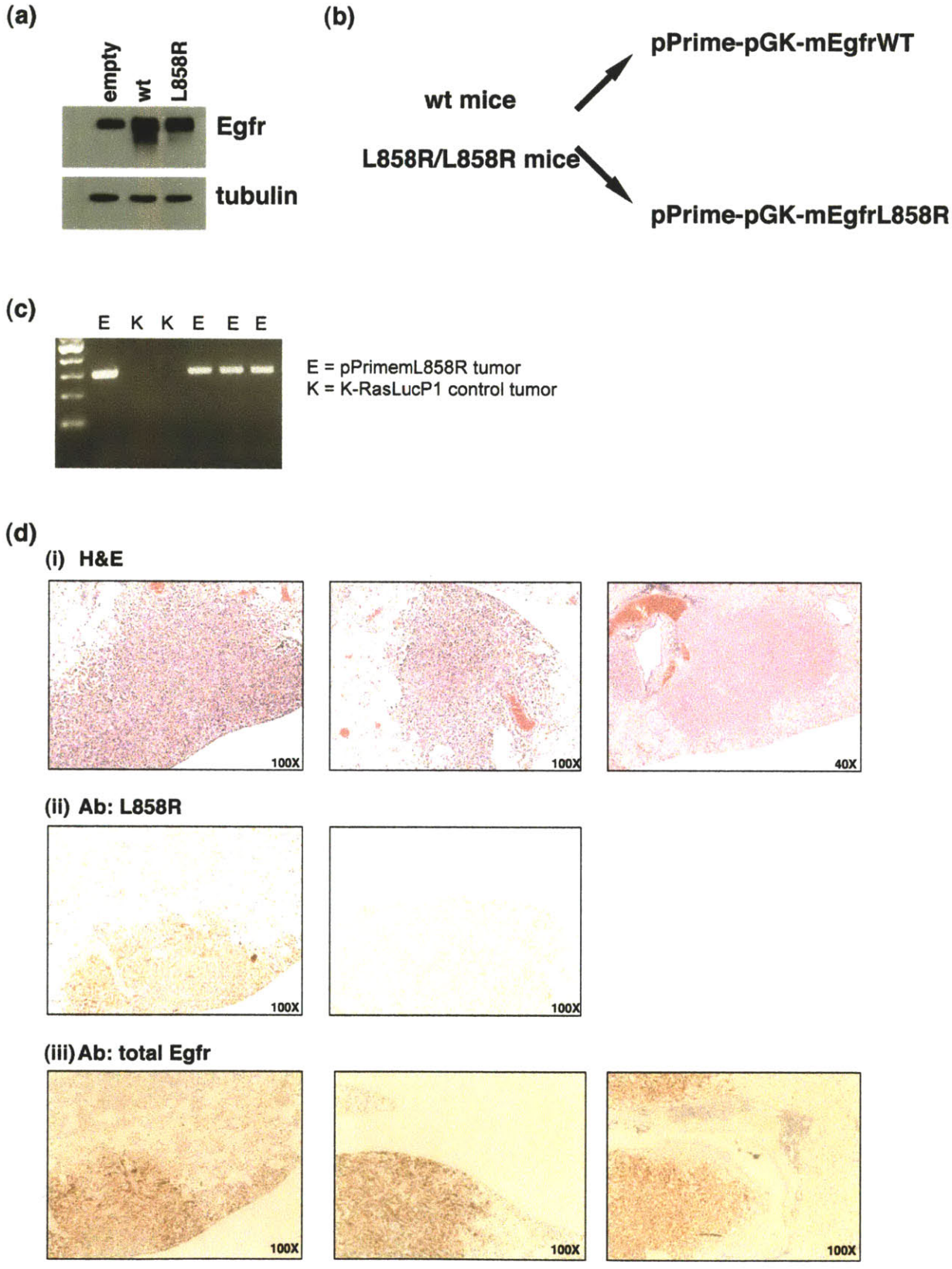

(a)
empty
wt
L858R
Egfr
tubulin
(b)
wt mice
L858R/L858R mice
pPrime-pGK-mEgfrWT
pPrime-pGK-mEgfrL858R
(c)
E K K E E E
E = pPrimemL858R tumor
K = K-RasLucP1 control tumor
(d)
(i) H&E
100X
100X
40X
(ii) Ab: L858R
100X
100X
(iii) Ab: total Egfr
100X
100X
100X

Figure 3. mEgfr^{L858R} can induce tumors in the lung when expressed from a lentivirus.

(a) MEFs were infected with lentiviruses expressing either WT or L858R Egfr cDNA and lysates were blotted for total Egfr confirming overexpression in both cases. **(b)** WT and L858R/L858R mice were infected with these lentiviruses as shown. **(c)** LCM analysis on tumors that arose in wild-type mice with the pPrime-Pgk-mEgfrL858R virus confirming the presence of the mutant cDNA. K=control K-RasG12D lung tumors that should be negative for the mutant cDNA. **(d)** Tumors that arose with the pPrime-Pgk-mEgfrL858R virus (i) H&E stained sections of lung tumors that arose using these viruses. All images are taken at 100X except where indicated. (ii) Tumors stain positive with the L858R specific antibody. (iii) Tumors stain positive for Egfr.

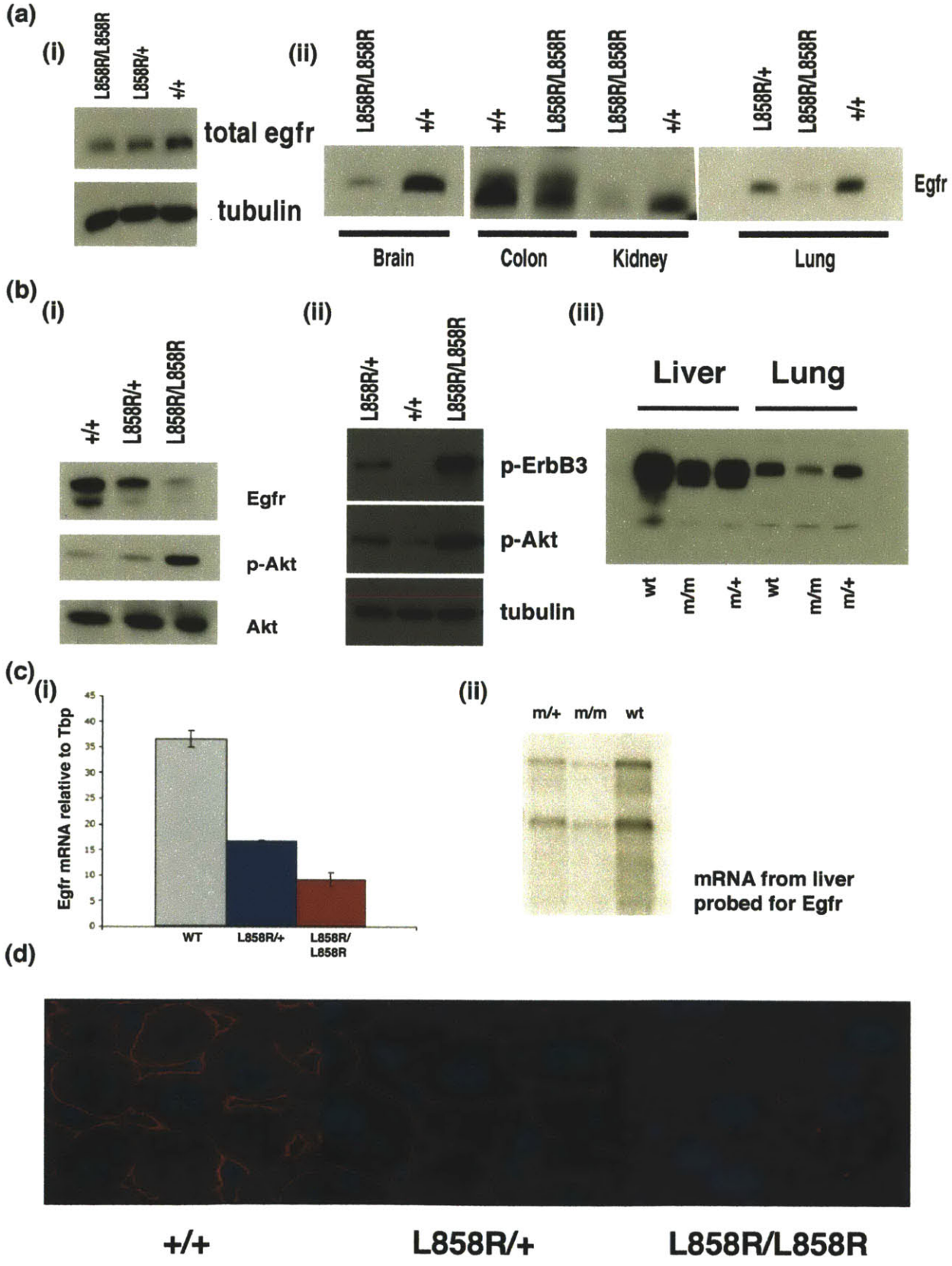
this could be for technical reasons associated with achieving sufficient expression from lentiviruses in the lung.

Activity dependent downregulation of mutant Egfr

Upon closer examination of tissues from the germline mutant mice we observed that the mutant receptor was expressed at much lower levels than the wild-type. This was true in MEFs (Figure 4A, i) and in all other tissues examined (Figure 4A, ii), with the receptor levels of the heterozygote showing intermediate expression. Although the differences in MEFs were not as striking as those found in tissues, they were reproducible across many independent MEF preparations. The livers of the germline mutant mice appear larger than those of wild-type littermates and so we interrogated some signaling pathways downstream of Egfr in these samples. Despite a large reduction in Egfr levels in the homozygous mutant mice a high level of p-Akt was found in these livers (Figure 4B, i), and this correlated with a high level of p-ErbB3, the known mediator of mutant EGFR induced Akt activation (Figure 4B, ii). This activation of the Akt pathway was not observed in any other tissue, including the lung. One possibility is that the higher basal levels of Egfr expression found in the liver compared to the lung (as shown for comparative purposes in Figure 4B, iii) is required to reach a threshold needed to activate downstream signaling. The germline mutant mice develop low-grade hepatomas with long latency (18months) and while it is possible that this induction of p-ErbB3/p-Akt signaling is involved in this we have not investigated this further. Unlike other tissues and MEFs where the levels of Egfr mRNA expression is equivalent among the genotypes (data not shown) there is a significant decrease in Egfr mRNA in both the heterozygous and homozygous germline mutant livers, as assessed by both qPCR (Figure 4C, i) and northern blotting (Figure 4C, ii). The protein downregulation was also confirmed by Egfr immunofluorescence on liver sections from each of the genotypes (Figure 4D).

We wanted to address whether the downregulation in mutant Egfr was dependent on the kinase activity of the receptor. To address this we treated both wild-type and

L858R/L858R mutant MEFs with Tarceva and monitored for changes in Egfr protein levels. Mutant Egfr levels were rapidly restored to wild-type levels upon kinase inhibition (Figure 4E); confirming that the decreased receptor levels were indeed a consequence of activity-dependent downregulation. We also wished to determine whether the proteasome was involved in mutant receptor degradation. To this end, we treated wild-type and mutant MEFs with MG132, a broad-spectrum proteasome inhibitor, and found that mutant Egfr levels were restored upon proteasome inhibition (Figure 4F). Both of these results suggest that mutant receptor mediated signaling is activating some form of feedback on the receptor, leading to its degradation. Although we had previously found only minimal differences in signaling between wild-type and mutant Egfr MEFs we decided to examine whether there was differential recruitment of adaptor proteins to the receptor, in an attempt to understand how a negative feedback signal might be initiated. We carried out a series of co-immunoprecipitation experiments in MEFs and found that there is a slight increase in the recruitment of adaptor proteins such as Shc and Grb2 to the mutant receptor (Figure 4G).


(a)
(i)
L858R/L858R
L858R/+
+/+
total egfr
tubulin
(ii)
L858R/L858R
+/+
+/+
L858R/L858R
L858R/L858R
+/+
L858R/+
L858R/L858R
+/+
Egfr
Brain
Colon
Kidney
Lung
(b)
(i)
+/+
L858R/+
L858R/L858R
Egfr
p-Akt
Akt
(ii)
L858R/+
+/+
L858R/L858R
p-ErbB3
p-Akt
tubulin
(iii)
Liver
Lung
wt
m/m
m/+
wt
m/m
m/+
(c)
(i)
Egfr mRNA relative to Tbp
45
40
35
30
25
20
15
10
5
0
WT
L858R/+
L858R/
L858R
(ii)
m/+
m/m
wt
mRNA from liver
probed for Egfr
(d)
+/+
L858R/+
L858R/L858R

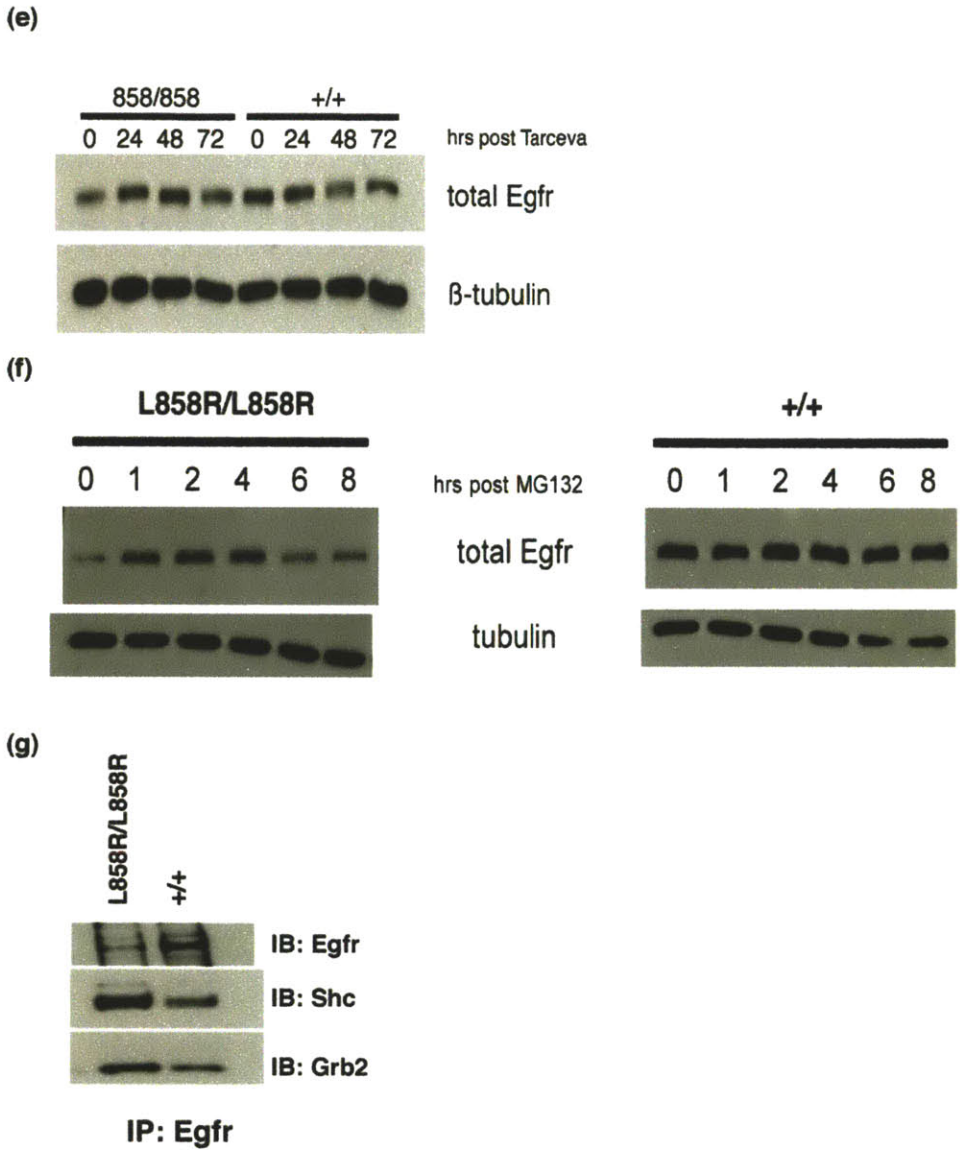


Figure 4. Downregulation of mutant Egfr is a universal feature of cells from Egfr^{L858R} mice

(a) Western blots for total Egfr protein levels in (i) MEFs and (ii) tissues from germline mutant mice showing downregulation of the mutant receptor. **(b)** Increased Akt pathway signaling in germline mutant liver (i) and (ii), and a comparison of total Egfr levels between liver and lung of wildtype and mutant mice (iii). **(c)** mRNA levels in livers of wt, L858R/+, and L858R/L858R mice were assessed by qPCR using Taqman probes (i) or by northern blotting (ii). **(d)** Immunofluorescence for total Egfr on paraffin section of mouse liver. Red = Egfr, Blue = DAPI **(e)** The treatment of MEFs with 5μM Tarceva for the indicated periods led to an upregulation in mutant Egfr protein levels. **(f)**. Treatment of MEFs with MG-132 also led to an increase in mutant Egfr protein levels. **(g)** Co-IPs on wild-type or L858R/L858R MEFs stimulated with EGF reveals an increase in the recruitment of adaptor proteins to the mutant receptor.

Discussion

The discovery of kinase domain mutations in NSCLC lung cancer patients who responded to TKIs led to the proposal that these lesions were the 'driver' mutation and likely were an early event in tumor formation. To investigate the causal role of $Egfr^{L858R}$ in NSCLC we generated a conditional and germline knock-in allele in the mouse. Based on the human data and the tumor phenotype of a previous model of mutant K-Ras that was generated in the lab, we assumed that tumors would develop, and that potentially the germline version of the allele would be embryonic lethal due to aberrant Egfr signaling during development. The absence of tumors and the viability of the germline mice were even more surprising given the robust tumor phenotype seen by other groups using doxycycline-inducible transgenics. Our initial expectation was that the $Egfr^{LSL\text{-}L858R}$ mice would develop tumors, but with a longer latency than the transgenics. With that in mind we attempted to accelerate the process by incorporating a range of tumor suppressor alleles into the model; these tumor suppressors had either been found to be deleted in EGFR mutant human tumors, or in the case of Nf2, were known to be involved in regulating EGFR signaling (Curto et al., 2007). The continued lack of a tumor phenotype presented the real possibility that there was a threshold level of Egfr needed for tumor formation and that it was simply the case that our knock-in allele was below that threshold, while the transgenics were fortuitously above it.

The initial identification of the mutations was followed by a number of reports documenting amplification of the mutant allele in approximately 70% of EGFR mutant tumors (Soh et al., 2009; Takano et al., 2005). This would seem to support a threshold effect for mutant EGFR and explain the difference between the mouse models. We

surmised though that a significant fraction of patients had mutation but no gene amplification and must therefore have acquired some other modification that our mouse model is lacking. When we went back to examine Egfr expression levels in our germline model we noticed a striking downregulation of the mutant receptor in all tissues and in MEFs derived from these animals. We found this downregulation to require the kinase activity of the receptor and to be mediated by the proteasome, at least in MEFs. Apart from the liver we found that signaling pathways downstream of Egfr were mostly unchanged, even in response to Egf stimulation in MEFs. This result actually confirms that the mutant receptor is hyperactive, as per receptor there is actually more signaling activity, but it is not manifest due to the lower levels of expression. This result is reminiscent of that seen with Dsk5, a germline *Egfr* mutation found in an ENU screen (Fitch et al., 2003), and that also happens to be one of the more rare mutations found in NSCLC patients who respond to TKIs.

The dramatic downregulation of mutant Egfr in our model altered our thinking about how Egfr mutant tumors might develop and the different stages of the disease that the knock-in model and the transgenic models represent. This is described in the model shown in Figure 5. Gene amplification is most often thought of as a means to increase levels of a protein beyond their normal range, but our data may suggest that the amplification observed in EGFR mutant NSCLC patients is more a means to counteract the downregulation that is induced upon mutant receptor expression, than to increase levels per se. If this downregulation is indeed a component of *EGFR* mutant human tumors then there are at least two routes by which a cell that harbors an *EGFR* mutation can develop into a tumor. The most common is of course to amplify the locus

and in this way overwhelm the downregulation pathway components, and cross the signaling threshold required to initiate tumor formation. The dox-inducible transgenic alleles model this final stage of tumorigenesis, as does the lentiviral delivery system we used; however, most of the evidence supports mutation preceding amplification (Tang et al., 2005; Tang et al., 2008) and with their rapid overexpression of mutant EGFR they fail to capture events that occur in between mutation and amplification, the relative importance of which we would probably be wise not to discount.

The other route to tumor formation for cells harboring *EGFR* mutations would be to disable the negative feedback mechanisms that are maintaining receptor expression at lower levels than required for tumor formation. With the advent of tumor resequencing efforts it would be interesting to compare EGFR mutant tumors with amplification to those without, and to look for alterations in genes that might mediate this feedback. Our knock-in allele of $Egfr^{L858R}$ models the initiating event and the downregulation that suppresses the oncogenic potential of this mutation. These two partially independent routes to EGFR mutant tumor formation may in fact generate tumors that both harbor oncogenic EGFR but are fundamentally different in terms of the ERBB signaling network. A recent analysis on alterations in the binding of adaptor proteins to EGFR as receptor levels are increased (or at least simulated to increase) revealed that significant quantitative and qualitative changes in binding are associated with a shift in EGFR expression levels (Jones et al., 2006). This raises the prospect that EGFR mutant patients can be further subdivided, and that this differential path to tumor formation may lead to variations in therapeutic response or in secondary modes of resistance to TKIs.

The bulk of this thesis is devoted to tackling the two major unknown areas outlined in the model in Figure 5. Chapter 3 focuses on our efforts to try to understand the negative feedback loop that is responsible for mutant Egfr downregulation in our model, with a view to understanding how *EGFR* mutant tumors without amplification might develop. Chapter 4 tackles the technical challenge of trying to engineer a mouse model of targeted amplification of mutant *Egfr*. This was an effort to try and develop a model that would allow us to understand the transition between single-copy mutant receptor and amplification, and interrogate how the alterations that occur during this transition may affect tumor progression or therapeutic response.

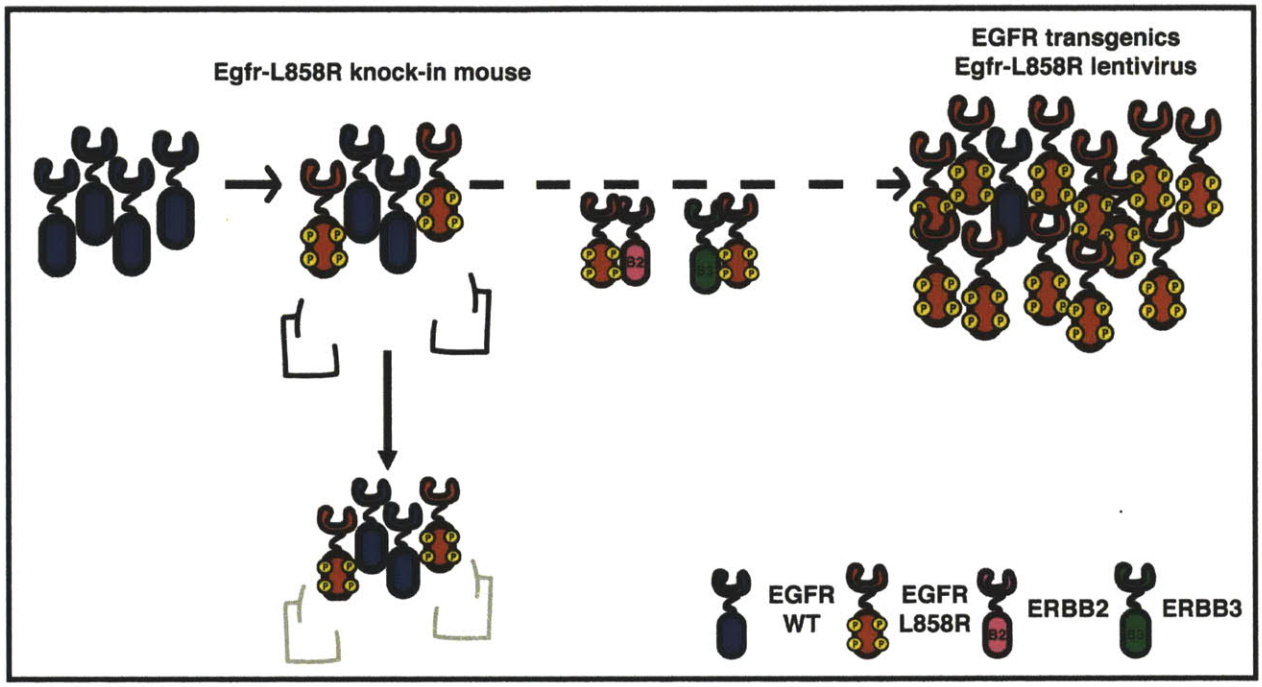


Figure 5. A potential model representing the evolution of EGFR mutant NSCLC, including a role for amplification and the potential contribution of other ERBB family members such as ERBB2 and ERBB3, along with the possibility that negative feedback control mechanisms restrain signaling from the mutant receptor, and disabling these mechanisms may represent another route to tumor formation.

Materials and Methods

Egfr co-immunoprecipitation protocol. This protocol was adapted from M. Curto (MGH). Briefly, serum-starved cells were treated with EGF for 10mins and then scraped in cold-PBS, pelleted and resuspended in a hypotonic buffer (10mM KCl, 1.5mM $MgCl_2$, 10mM $Tris_{7.4}$, 1mM EDTA, 1mM EGTA, phosphatase and protease inhibitors) for 30mins on ice. Cells were disrupted with a Dounce, breakage was confirmed under a scope, and the suspension was ultracentrifuges for 1hr at 4°C at 100000xg. The membrane-containing pellet was solubilized in a triton-lysis buffer (1% Triton X-100, 60mM n-octyl-beta-D-glucopyranoside, 50mM $Tris_{7.4}$, 140mM NaCl, 1mM EDTA, 1mM EGTA, phosphatase and protease inhibitors) and incubated on ice for 30-40 mins. This was followed by a 10min spin at 14000g and the supernatant was used for the IP. ProteinA beads were used for the IP and 7μg of antibody (Ab-17) was used per IP. After the final IP wash the beads were pelleted and resuspended in the following loading buffer: 2M urea, β-mercaptoethanol, 5X sample buffer, and TLB. ProteinA-HRP was used as the secondary antibody for western blotting to reduce the background.

Lentivirus preparation. For lentivirus preparation, 293FT cells were plated and 24hrs later, with the cells at 50% confluency, they were transfected with a 4:3:1 mix of vector:delta8.2:VSVg combined with TransIT-LT1 transfection reagent (MirusBio). Viral supernatant was harvested and filtered at 48hrs and 72hrs post-transfection, and added directly to cells in combination with polybrene (10μg/ml). For *in vivo* experiments lentivirus was prepared using 15cm dishes of 293FT cells and the 48hr and 72hr supernatants were pooled, and ultracentrifuged for 2hrs, at 4°C and 25,000 rpm. The viral pellet from each 15cm plate was resuspended in 200μl of 1X HBSS, pH 7.4 and titered for Cre expression as previously described (DuPage et al., 2009).

Virus that was to be administered within 4-5 days was stored at 4°C, otherwise it was aliquoted and stored at -80°C until needed.

Targeting construct design. All constructs were made using recombineering technology. The FRTneoFRT plasmid used is available from NCI recombineering division. BAC clone RP23-263C13 was purchased and found to contain the Egfr locus. A SalI site was recombineered into intron 1 and the targeting arms recombineered out into pKS-DTA. The LSL was then cloned into this SalI site using XhoI. Once the FRT-neo-FRT and the mutation were recombineered onto the BAC the targeting region was removed into pKS-DTA. v26.2 ES cells were targeted in both cases.

Protein extraction and immunoblots. Cells were always lysed on ice following two washes with ice-cold PBS. The extraction buffer used was as follows: 10% Triton, 50mM $Tris_{7.4}$, 150mM NaCl, 0.5% sodium-deoxycholate, 0.1% SDS, 1mM DTT, 1mM EDTA, supplemented with Mini-protease inhibitor tablets (Roche) and phosphatase inhibitor cocktails 1 and 2 (Sigma). Lysates were rocked at 4°C for 15mins and clarified by a 14,000rpm spin, 15mins, 4°C. Tissues were processed in a similar manner on ice, using either a razor blade or a small dounce to complete homogenization. Supernatants were quantitated using the BCA assay (Pierce), and diluted with a standard sample buffer containing β-mercaptoethanol. Lysates were run on SDS-PAGE gels, made in house or purchased from Invitrogen, and transferred to PVDF membrane. Antibodies used included: β-tubulin (Sigma T4026; CST #2128), Egfr (sc-03, Santacruz; 06-847, upstate; Ab-17, Labvision), L858R (W.Pao. MSKCC), p-ErbB3 (CST), p-AktSer473 (CST), Shc (BD Biosciences), Grb2 (BD Biosciences),

RNA extraction, cDNA preparation and qPCR. RNA was isolated using either the Qiashredder and RNeasy Mini Kit (Qiagen) or with a standard Trizol extraction protocol. cDNA was generated using 1μg of RNA with the Superscript III kit (Invitrogen) and random hexamers.

qPCR was carried out with Taqman reagents using an ABI PRISM 7000 Sequence Detection System Thermo Cycler (Applied Biosystems). Taqman probes used were: mEgfr: Mm00433023_m1, mTbp: Mm00446973_m1. Northern blotting was carried out according to a standard protocol as detailed in Qiagen RNeasy handbook.

EGF internalization assays. EGF internalization assays were carried out by M. Lazzara as described in (Lazzara et al., 2010).

LCM on tumor sections. LCM was carried out on paraffin sections as detailed in (Gidekel Friedlander et al., 2009) and DNA isolated using a kit from Arcturus.

Mice. *Trp53*$^{FL/FL}$ mice were provided by A. Berns (Netherlands Cancer Institute). *Nf2*$^{FL/FL}$ mice were generated in our lab, as was the *R26-CreER* allele. Adenoviral and lentiviral intratracheal infections was carried out as previously described (DuPage et al., 2009). Tamoxifen (Sigma) was resuspended in corn oil (Sigma) at 10mg/ml and intraperitoneal injections administered at 4.5mg/40g of body weight every two days for 8 days. Animal studies were approved by the Massachusetts Institute of Technology's Committee for Animal Care and conducted in compliance with Animal Welfare Act Regulations and other federal statutes relating to animals and experiments involving animals, and adheres to the principles set forth in the 1996 National Research Council Guide for Care and Use of Laboratory animals (institutional animal welfare assurance number, A-3125-01).

Cell culture. MEFs were generated from E13.5 embryos, except for the LSL/LSL MEFs, which were generated at E11.5-E12.5, and cultured in DMEM (DME, 10% FBS, 2mM glutamine, 1X Pen/Strep). mEGF was purchased from Sigma, MG-132 from EMD, and Tarceva was obtained from Genentech.

Histology and immunohistochemistry. Tissues were fixed in 10% formalin overnight, transferred to 70% ethanol and processed for histology. Paraffin sections were dewaxed, followed by antigen-retrieval using 10mM citrate buffer (pH6.0). Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide, slides were washed in PBS/0.1% Tween (PBS-T), and blocked in 10% normal goat serum in PBS-T for 1hr at RT. Sections were incubated overnight with either an Egfr antibody (Upstate, 06-847) or an L858R specific antibody (W. Pao) at 4°C. In both cases a biotinylated goat anti-rabbit secondary was used and signal was detected with a DAB kit (Vectastain). Slides were counterstained with H&E and then coverslipped.

Immunofluorescence. Paraffin sections were dewaxed, followed by antigen-retrieval using 10mM citrate buffer (ph6.0). Sections were blocked with 10% normal goat serum in PBS-T for 1hr at RT and incubated overnight at 4°C with a 1:200 dilution of the Egfr antibody (Upstate, 06-847) in the blocking solution. Slides were washed in PBS-T and incubated for 45mins with an AlexaFluor 594 donkey anti-rabbit secondary antibody (Invitrogen), and after addition of DAPI slides were mounted and imaged using a DeltaVision microscope.

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Chapter 3.

Negative regulation constrains the oncogenic potential of mutant EGFR.

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Keara Lane performed all experiments in the laboratory of Tyler Jacks, apart from the internalization assays that were carried out by Matthew Lazzara in the laboratory of Doug Lauffenburger.

Abstract

Multiple layers of positive and negative feedback control converge on signaling pathways to ensure an appropriate output in response to a stimulus. The role of oncogenes is typically thought of in terms of increasing the output from a given position in a signaling pathway, but the role of the associated negative feedback loops, while no doubt important, has been afforded scant attention. Recent studies have highlighted the existence of such feedback mechanisms in established tumors and the integral role they play in shaping the signaling network, with a corresponding appreciation for how these feedback loops can profoundly influence therapeutic response. The capacity of such negative regulators to modulate the oncogenic potential of a mutant protein in the context of tumor initiation has however rarely been examined. We had previously documented diminished levels of Egfr protein in tissues and MEFs from germline Egfr mutant mice, and in this study sought to elucidate the underlying mechanism. We unmask the role of a known Egfr negative regulator, Mig-6, in the suppression of mutant Egfr signaling both in MEFs and skin; deletion of Mig-6 fails however to alter Egfr protein levels, or elicit tumor formation in the lung, precluding the possibility that Mig-6 is responsible for the observed mutant receptor downregulation. To probe candidate proteins involved in this feedback we establish a cell culture system based on inducible Egfr expression; this strategy strikingly recapitulates the aforementioned degradation of the mutant receptor, but now the kinetics are such that the system is amenable to modification by shRNAs/cDNAs. Using this approach we establish a role for ERK pathway signaling, and specifically DUSP6, in receptor degradation, potentially mediated through phosphorylation of T693 of Egfr, and solidify a role for the E3 ligase, CULLIN5, in receptor degradation. The existence of such negative feedback loops, together with their profound effects, both on mutant receptor levels and the oncogenic potential of this lesion, underscore the negation of this barrier as a requisite step in mutant Egfr tumor evolution, and may be the basis for the coincident occurrence of Egfr mutation and gene amplification in NSCLC.

Introduction

The complexity of RTK signaling pathways, which transmit extracellular cues into intracellular responses, requires tight regulation to ensure cells respond accordingly, and only in the presence of an appropriate signal. The metazoan expansion of the RTKs and their ligands was accompanied by a coordinate increase in feedback control mechanisms to terminate signaling, establish a refractory period, and require re-stimulation prior to the initiation of another response (Amit et al., 2007b). Cellular homeostasis requires a balance between positive and negative signaling, and this equilibrium is most often shifted in favor of the former during tumorigenesis. Oncogenes, in concert with increased flux through downstream pathways, typically either overwhelm or subvert negative control mechanisms to facilitate tumor induction and progression. The reduced complexity of the EGFR signaling pathway in both *C.elegans* and *D.melanogaster* restricts its ability to adapt signaling to contend with any signaling aberration. The expansion of the ERBB family in vertebrates increased the signaling capacity of this now expanded, interconnected network, and with it the propensity to tolerate perturbations to the pathways, thus markedly increasing the robustness of the signaling output. The tumorigenic impact of mutations at particular levels of the EGFR pathway may therefore be distinct and may have a differential dependence on disabling negative feedback control mechanisms.

The advent of targeted therapy is based on the premise that increased signaling through a pathway will confer sensitivity to its inhibition. This often holds true if the protein under scrutiny represents the so-called 'driver' mutation, but in many contexts increased steady-state signaling is not an accurate reflection of the complex underlying signaling dynamics that dictate sensitivity. Uniformly increased levels of p-ERK in tumors with alterations in any of RTKs, RAS, or B-RAF, fails to be a pertinent predictive marker for clinical response to MEK inhibition, with only B-RAF mutant tumors demonstrating any appreciable sensitivity (Solit et al., 2006). The sensitivity of B-RAF^{V600E} tumors to this inhibitor is a consequence of the canonical feedback regulation on MEK no longer being operational in these tumors. This is reflected in increased basal levels of p-MEK,

which manifests as an increased ERK pathway signaling output, assessed both by induction of transcriptional targets downstream of p-ERK and by a coincident reliance on the MAPK pathway for survival (Pratilas et al., 2009). In non-B-RAF mutant tumors p-MEK remains sensitive to feedback inhibition and the signaling flux through the pathway is diminished, as evidenced by a failure to engage a similar ERK mediated transcriptional response as observed in B-RAF mutant tumors. An analysis of the steady-state p-ERK levels in tumors misrepresents the reliance of tumors on this pathway, and only through uncovering the dynamics of the positive and negative feedback regulation, together with their overall impact on each node of the pathway, can true dependence be determined. The use of small molecule inhibitors to assess pathway sensitivity has serendipitously unmasked the intricate network of feedback mechanisms that are actively employed to regulate signaling in tumor cells, and has underscored the previously undocumented perilous potential of such inhibitors to reactivate pathways in certain tumor genotypes (Chandarlapaty et al., 2011; Cloughesy et al., 2008; Serra et al., 2011). Feedback mechanisms remain active in late stage tumors, but their role in shaping the initial stages of tumor formation, or indeed in susceptibility to tumor initiation, have been examined in a limited fashion.

Feedback mechanisms are an essential component of normal cellular homeostasis, and though it is apparent that disabling such regulators may increase already deregulated signaling in established tumors, evidence for a causal role in tumor formation or progression is mostly absent. The EGFR signaling pathway involves multiple layers of feedback regulation, from the acute recruitment of CBL, an E3 ligase that terminates RTK signaling, to the transcriptional induction of feedback regulators such as MIG-6 and SPRY2, along with other immediate early genes (IEGs) and delayed early genes (DEGs) known to regulate signaling output. *CBL* has recently been found to be mutated in CML and NSCLC (Sargin et al., 2007; Tan et al., 2010), and a Y1045F mutation in EGFRvIII decreases CBL binding and increases the transforming capacity of this oncogene (Davies et al., 2006). A gene-set comprised of negative feedback

regulators elicited upon EGF stimulation of HeLa cells was recently found to be downregulated in many tumors and this correlated with decreased survival (Amit et al., 2007a). While this has potential prognostic value it, along with many studies altering the status of negative regulators in established human cell lines, fails to address whether negative regulators of the EGFR signaling cascade directly influence tumor formation. Mouse models involving knockouts of some of these regulators have begun to address this question. Constitutive knockouts of *Lrig1* and *Spry2* fail to develop tumors, while *Mig-6* null mice are sensitive to chemically induced papillomas and melanomas (Ferby et al., 2006; Suzuki et al., 2002; Taketomi et al., 2005). Due to their pivotal role in regulating signaling during development, the deletion of many of these negative regulators leads to a partially penetrant embryonic lethality, and as such conditional null alleles will be essential to assess whether they have a veritable role in tumor formation.

In the context of oncogenic K-Ras expression in the lung, conditional *Spry2* deletion leads to an increase in both tumor number and size, and an upregulation in p-Erk (Shaw et al., 2007). In contrast to transgenic models that ectopically express oncogenic K-Ras, the tumors that arise through expression of a single copy of the mutant allele from the endogenous locus fail to stain positive for p-Erk by IHC, and MEFs from these mice show a paradoxical decrease in p-Erk and p-Akt signaling post cre-mediated activation of the allele (Fisher et al., 2001; Jackson et al., 2001; Tuveson et al., 2004). Recent analysis has revealed an upregulation of p-Erk in late-stage tumors when this allele is combined with loss of p53, and this further correlates with either amplification of *K-Ras* or abrogation of negative regulators of Erk signaling such as Spry2 (Feldser et al., 2010; Junttila et al., 2010). While progression is impaired, tumors do arise upon activation of single copy oncogenic K-Ras despite the presence of feedback regulation; nonetheless, determining the role of this feedback in shaping tumor formation will be critical to understanding the sensitivity of pathways to therapeutic intervention.

An analysis of tissues from the germline $Egfr^{L858R}$ mutant mice, along with MEFs derived from these mice, found significantly decreased Egfr protein levels as compared to wild-type. We

have documented that this downregulation is dependent on Egfr kinase activity, and the ability of the Egfr pathway to tolerate this activating mutation by virtue of a decrease in receptor expression levels, exemplifies the adaptability of the ERBB signaling network and the important role of feedback in controlling it. Together with the lack of a tumor phenotype in the mutant mice this downregulation has led us to conclude that a threshold level of oncogenic Egfr expression is required for tumor initiation, and that the concurrent amplification observed in EGFR mutant human tumors is essentially one mechanism used to counteract downregulation of the mutant kinase. We sought to understand the molecular mechanisms underlying the downregulation of mutant Egfr in this model. The apparent adaptability of the network to this mutation has led us to develop an inducible system with which to explore the early events that initiate mutant Egfr downregulation and to further our understanding of the natural course of EGFR mutant tumor evolution.

Results

Marginal upregulation of known negative regulators in mutant Egfr expressing cells

Our initial characterization of the L858R knock-in mouse led us to conclude that the mutant receptor was subject to activation-dependent downregulation (Chapter 2). We sought to understand the molecular mechanism underlying this feedback, and we began by assessing the expression level of known negative regulators in both tissues and cells derived from the germline mutant mice. We selected proteins that had been linked to direct regulation of either levels, or activity, of EGFR and this left us with five candidates, LRIG1, SPROUTY2, MIG-6, GCF2, and AP2-α (Gur et al., 2004; Hackel et al., 2001; Haglund et al., 2005; Johannessen et al., 2006; Rikiyama et al., 2003). LRIG1, SPROUTY2 and MIG-6 have all been shown to be transcriptionally-induced by EGF stimulation, and along with AP2-α, they either regulate EGFR internalization and degradation, or impair dimerization and activation of downstream signaling. GCF-2 is a transcriptional repressor but was included in the analysis due to the decreased Egfr mRNA levels detected in the liver of germline mutant mice. cDNA was prepared from the livers of a panel of wild-type, L858R/+, and L858R/L858R mice and we found that the only changes were an upregulation in Mig-6 and Gcf2 expression levels in the mutant mice as compared to wild-type controls (Figure 1A). We carried out a similar analysis on wild-type and L858R/L858R mutant MEFs and while there was an upregulation in Lrig1, Gcf2 and Mig6 levels, the differences were marginal (Figure 1B). The expression level of Egfr in MEFs is lower than that of most epithelial tissues and many tumor cell lines, and as such we sought to analyze the induction of negative regulators in the context of increased levels of Egfr. We infected NIH-3T3 cells with a retrovirus, that expressed either murine $Egfr^{WT}$, murine $Egfr^{L858R}$, or human $EGFR^{L858R}$, selected a pure population and harvested RNA. As shown in Figure 1C, Lrig1, Gcf2 and Spry2 were preferentially induced by the mutant receptor, but the degree of induction was low and the results of our analysis did not overlap with our previous results from either liver or

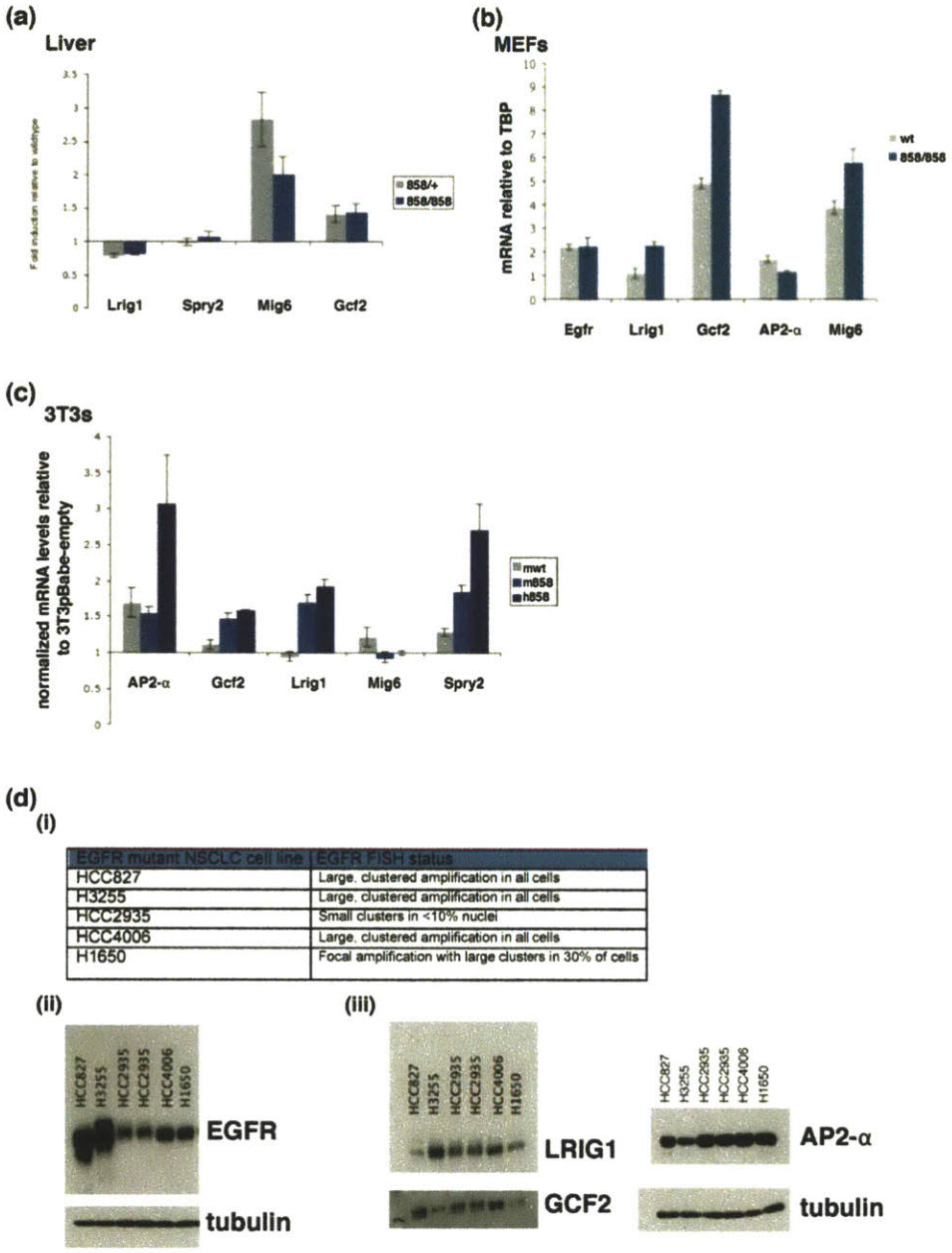


Figure 1. Marginal upregulation of negative regulators in cells expressing Egfr-L858R.

(a) qPCR, using Taqman probes, was carried out on RNA isolated from the liver of wild-type, L858R/+, and L858R/L858R mice, and the results are represented as fold induction over wild-type. **(b)** qPCR using Taqman probes on RNA from wild-type and L858R/L858R MEFs. **(c)** qPCR on RNA isolated from NIH-3T3 cells overexpressing $mEgfr^{WT}$, $mEgfr^{L858R}$, or $hEGFR^{L858R}$. Results are expressed as fold-change relative to cells infected with empty vector. **(d)** (i) Reported amplification status of human EGFR mutant NSCLC cell lines. Western blot for (ii) EGFR levels, and (iii) for candidate negative regulators of EGFR in human NSCLC cell lines.

MEFs, leaving us without an obvious candidate to pursue further. A number of NSCLC cell lines exist that are derived from EGFR mutant lung tumors, and these have been previously characterized using FISH as to their EGFR amplification status (Helfrich et al., 2006)(Figure 1D, i). We hypothesized that in cell lines without significant EGFR amplification, such as HCC2935, a putative negative regulator may be lost or downregulated when compared to lines such as H3255, known to have high-level amplification of EGFR (Tracy et al., 2004). We found no obvious correlation between EGFR amplification status, EGFR protein level, and the expression of any of the negative regulators (Figure 1D, ii, iii).

cDNA expression is sufficient to recapitulate receptor downregulation

With the exception of the liver, we had established that the downregulation of the mutant receptor in L858R germline mice was mediated by a post-transcriptional mechanism. We decided to investigate whether there was any role for UTRs or other non-coding elements in this downregulation; this would determine whether we could use ectopic expression systems to further our understanding of the mechanism. To this end, we infected wild-type MEFs with an MSCV retrovirus expressing the murine cDNA of $Egfr^{WT}$, $Egfr^{L858R}$, $Egfr^{T790M}$, or $Egfr^{T790M\text{-}L858R}$, and selected a population of puromycin-resistant cells for further analysis (Figure 2A). We determined the mRNA expression levels in these cells and found Egfr to be uniformly overexpressed, with levels in $Egfr^{T790M\text{-}L858R}$ expressing cells more elevated than the others (Figure 2B, i). However, when we carefully examined the Egfr protein levels using western blotting, we found that in both the $Egfr^{L858R}$ and the $Egfr^{T790M\text{-}L858R}$ populations the receptor was expressed at lower levels compared to the other non-L858R expressing cells (Figure 2B, ii). Having validated that ectopic expression of the cDNA could recapitulate the downregulation previously seen in the Egfr mutant MEFs and mice, we wanted to confirm that these mutant over-expressor cells still retained features of the original mutant MEF lines. We chose to

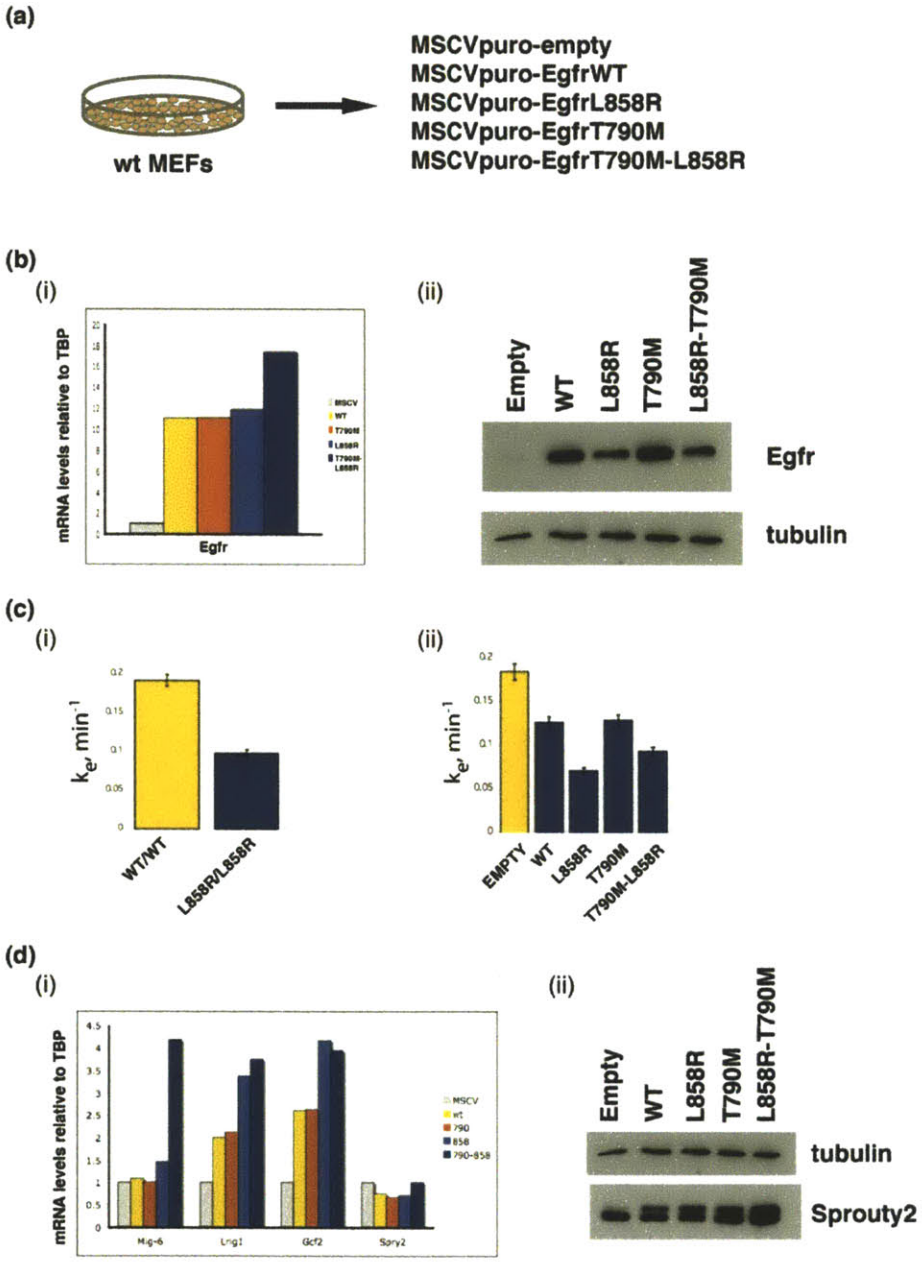


Figure 2. Ectopic expression of mutant Egfr cDNAs recapitulates the downregulation of mutant Egfr observed in germline mutant mice.

(a) The panel of Egfr mutant cDNAs as shown were expressed in wild-type MEFs using retroviral infection. **(b)** (i) qPCR for Egfr mRNA levels and (ii) western blotting for Egfr protein levels in wt MEFs expressing the indicated version of Egfr. **(c)** Rate constants for Egfr internalization (k_e) in response to EGF in (i) +/+ and L858R/L858R MEFs, and in (ii) wt MEFs overexpressing the indicated receptor. **(d)** (i) qPCR for negative regulators on RNA isolated from these Egfr-overexpressing MEFs, and (ii) a western blot for Spry2 expression in the same lines.

examine the Egfr internalization rate in response to stimulation with a low dose of Egf due to the highly quantitative nature of the readout and because a decreased internalization rate had already been shown to be a property of mutant-expressing NSCLC cell lines (Hendriks et al., 2006). We used wild-type and L858R/L858R mutant MEFs and showed that on an otherwise isogenic background a reduced internalization rate is indeed a property of the mutant receptor (Figure 2C, i), and was independent of the context of the other mutations found in cancer cell lines. We next tested the internalization rate in the wild-type MEFs ectopically expressing the panel of Egfr receptors, and found that Egfr overexpression alone was able to promote a decrease in the internalization rate, but that the presence of the L858R mutation led to a further decrease (Figure 2C, ii), mirroring the original MEF results. Additionally we found the internalization rate for the double mutant receptor, T790M-L858R, to be intermediate between the single L858R mutant and either the wild-type or T790M mutant (Figure 2C, ii). We have not investigated this further, but it supports the published data of T790M having a function beyond conferring resistance to gefitinib or erlotinib, and this intermediate internalization rate may be a result of differential receptor phosphorylation and subsequent recruitment of effector proteins.

We now had a system that expressed high levels of the mutant receptor but where the downregulation mechanism was still intact. We next tested whether any of the known negative regulators were specifically induced by the mutant receptors. Our analysis failed to reveal any major differences in induction, apart from a T790M-L858R specific induction of Mig-6 and a possible phosphorylation of Sprouty2 upon overexpression of any of the Egfr variants (Figure 2D, i and ii). We noticed a trend towards increasing transcriptional induction of Lrig1 and Gcf2 in the L858R expressing cells, although the differences with $Egfr^{WT}$ were minimal. While this system revealed the restriction of the downregulation to expression of the cDNA, it failed to provide further insight as to the underlying mechanism.

Adjusting negative regulator levels in MEFs fails to upregulate mutant receptor levels

We reasoned that if one of our candidate negative regulators was responsible for the Egfr downregulation observed in MEFs we should be able to reverse this using shRNAs directed against these regulators. We designed and validated shRNAs (data not shown) for each of the regulators previously under consideration, along with new candidates such as Socs5 and ER-α that had recently been linked to EGFR regulation, and infected both wild-type and L858R/L858R MEFs using the same viral preparation. This analysis failed to reveal any effect of knockdown of these candidates on the ratio of wild-type to mutant receptor (Figure 3A). One interpretation of this data is that none of these regulators are involved in mutant receptor degradation, but it is also possible that the level of knockdown achieved is insufficient to elicit an effect, or that once the downregulation has been in place for a prolonged time period that it is difficult to reverse.

Ablation of regulators in vivo fails to induce tumor formation in the lung

We decided that ablation of these negative regulators in the context of acute expression of mutant Egfr in the lung would be the most opportune context in which to assess their role, if any, in the regulation of receptor levels. To this end, we began by cloning validated shRNAs against either Lrig1 or Mig-6 into a lentiviral vector containing a U6-shRNA-Pgk-Cre cassette, and infected wild-type and germline mutant mice in the context of $p53^{FL/FL}$ with these viruses. No tumors developed. However, this could be due to technical difficulties in achieving robust knockdown using shRNAs in the lung. We decided that complete ablation of any negative regulator of interest was desired. The *Spry2* floxed allele was available in the lab, and while the exact role of Spry2 in regulating Egfr expression remains unclear we crossed this allele into our conditional *Egfr* mutant strain, and infected the resulting mice with adenovirus-Cre. This also failed to elicit a tumor phenotype.

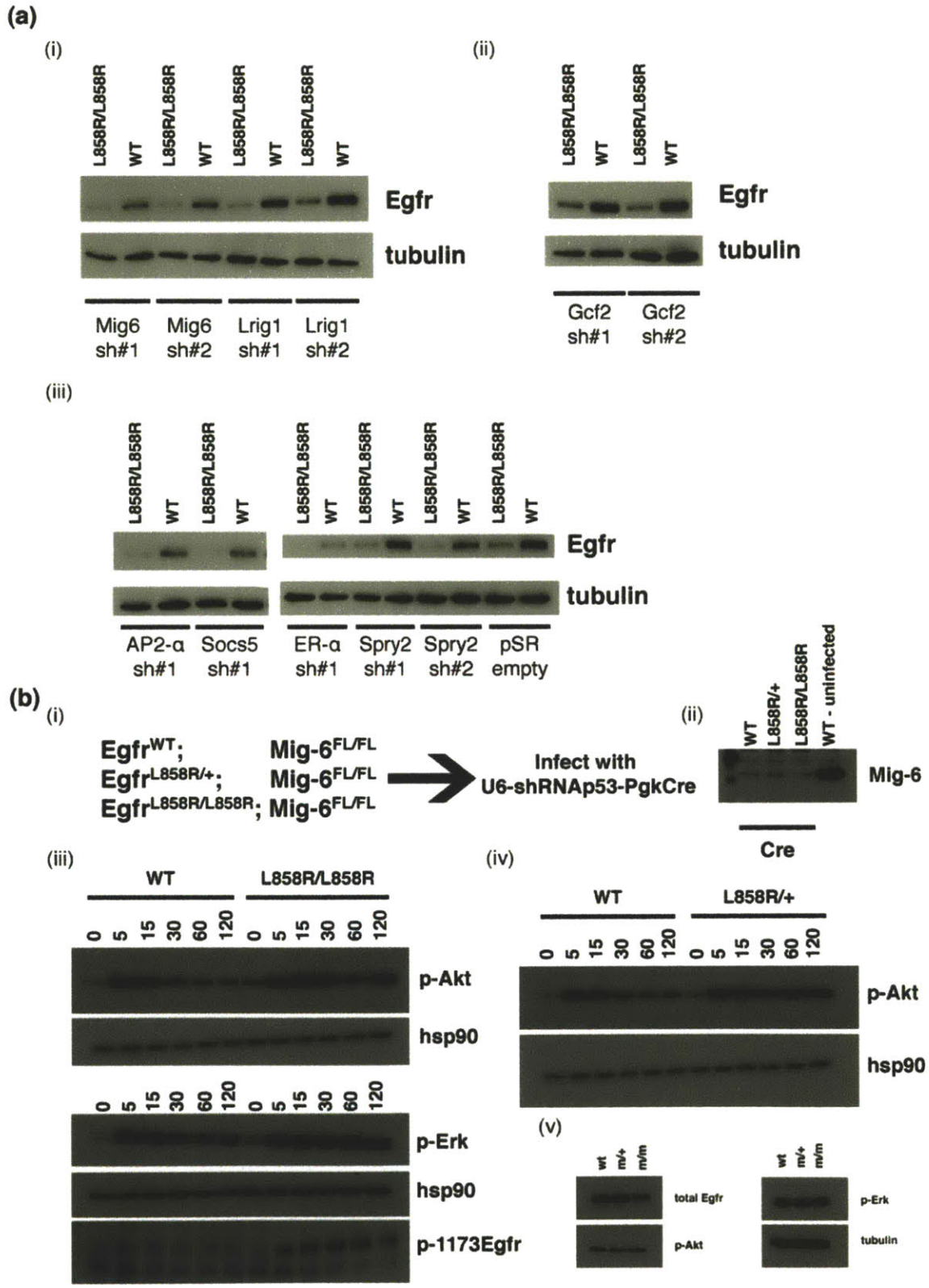

(a)
(i)
L858R/L858R
WT
Egfr
tubulin
Mig6 sh#1
Mig6 sh#2
Lrig1 sh#1
Lrig1 sh#2
(ii)
Gcf2 sh#1
Gcf2 sh#2
(iii)
AP2-α sh#1
Socs5 sh#1
ER-α sh#1
Spry2 sh#1
Spry2 sh#2
pSR empty
(b)
(i)
Egfr^WT; Mig-6^FL/FL
Egfr^L858R/+; Mig-6^FL/FL
Egfr^L858R/L858R; Mig-6^FL/FL
Infect with U6-shRNAp53-PgkCre
(ii)
WT
L858R/+
L858R/L858R
WT - uninfected
Mig-6
Cre
(iii)
WT
L858R/L858R
0 5 15 30 60 120
p-Akt
hsp90
p-Erk
p-1173Egfr
(iv)
L858R/+
(v)
wt
m/+
m/m
total Egfr
p-Akt
p-Erk
tubulin

Figure 3. With the exception of Mig-6, the modification of the expression levels of known negative regulators in MEFs has no effect on mutant Egfr.

(a) WT and L858R/L858R MEFs were infected with lentiviruses expressing the indicated shRNAs against candidate negative regulators, and the effect on Egfr protein levels monitored by western blotting. **(b)** (i) The panel of Mig-$6^{FL/FL}$ MEFs as indicated was infected with a U6-shRNAp53-Pgk-Cre lentivirus and (ii) loss of Mig-6 was confirmed by comparing Mig-6 levels in Cre treated and uninfected cells by western blotting. Serum-starved MEFs then underwent a time course of Egf stimulation and the activation of downstream signaling pathways was monitored using the antibodies indicated. Panel (iii) compares the effect of Mig-6 loss between $Egfr^{+/+}$ and $Egfr^{L858R/L858R}$ MEFs, while panel (iv) compares the effect of Mig-6 loss between $Egfr^{+/+}$ and $Egfr^{L858R/+}$ MEFs. (v) Signaling in Mig-6 deleted MEFs in 10% serum.

At the initiation of these experiments Mig-6 was the most well characterized of the Egfr negative regulators under study; moreover a knockout mouse had recently been shown to display hyperactive Egfr signaling and to develop a range of tumor types, including lung adenomas. We obtained a conditional *Mig-6* knockout allele as the pleiotropic effects of the straight knockout would potentially confound results, and make studies extending beyond 7-8 months impossible (Ferby et al., 2006; Jin et al., 2007). We crossed the germline *Egfr* mutant allele to the conditional *Mig-6* knockout and generated a panel of MEF lines as depicted in Figure 3B, (i). These cells were infected with a lentivirus carrying Cre recombinase along with an shRNA to p53 to immortalize the MEFs. Complete recombination was verified at the DNA level (data not shown) and by loss of the Mig-6 protein (Figure 3B, ii). We set out to determine whether loss of Mig-6 would alter the response of these cells to Egf stimulation. Serum-starved MEFs were stimulated with 100ng/ml Egf for the indicated times, and the status of downstream signaling pathways assessed by western blotting. L858R expressing MEFs, both hetero- and homozygous lines, displayed an extension in the duration of p-Akt and p-Erk signaling, and perhaps a modest increase in the initial response to EGF stimulation, although these bands have not been quantitated (Figure 3B, iii and iv). These data suggest that in the context of MEFs, mutant Egfr signaling in response to Egf may be constrained by Mig-6. However, we were unable to detect any alteration in Egfr expression levels in response to deletion of Mig-6 (Figure 3B, v) and basal signaling in 10% serum was similar among genotypes.

As a result of our analysis of Mig-6 ablation in MEFs, and along with published reports of the role of Mig-6 in inhibition of receptor signaling rather than induction of receptor ubiquitination and degradation (Hackel et al., 2001; Zhang et al., 2007), it seemed unlikely that Mig-6 was responsible for the decrease in mutant Egfr levels. It was still possible that ablation of Mig-6 in the lung would increase mutant receptor signaling analogous to what we had observed in MEFs, and that this would be sufficient to compensate for the decreased receptor levels, and initiate tumor formation. To determine if there was any functional consequence to deletion of Mig-6 in

the lung we infected a cohort of *Egfr*$^{LSL\text{-}L858R/+}$; *Mig-6*$^{FL/FL}$ and *Mig-6*$^{FL/FL}$ mice with adenovirus-Cre and aged them for 18 months. Surprisingly none of the mice, including the *Mig-6*$^{FL/FL}$ mice developed any tumors, in contrast to the observation of lung adenomas in a fraction of *Mig-6* straight knockout animals by 7-8 months (Ferby et al., 2006; Zhang et al., 2005). A similar finding was made by the group that constructed the conditional allele when they compared postnatal to embryonic deletion of *Mig-6* (Jin et al., 2009). It appears that the development of adenomas in the straight knockout is likely a consequence of the resulting defects in lung development rather than a direct role of Mig-6 in suppressing Egfr signaling.

In the process of these experiments we had wanted to determine whether deletion of *Mig-6* together with activation of mutant Egfr in the liver would lead to the development of advanced hepatomas or HCC, and combined the required alleles with *R26*$^{CreER/CreER}$ (Ventura et al., 2007). We administered tamoxifen to cohorts of mice and unexpectedly found that within 6-12 weeks all *Egfr*$^{L858R/L858R}$; *Mig-6*$^{FL/FL}$; *R26*$^{CreER/CreER}$ developed benign papillomas on any hairless skin surface, including ears, tail and mouth. None of the control mice developed these lesions in the timeframe of the experiment. Together with the results of extended signaling from MEFs this highlights the role Mig-6 plays in certain contexts in restraining mutant Egfr signaling. However, due to the general restriction of *EGFR* mutations to NSCLC, and the lack of a tumor phenotype upon *Mig-6* ablation in the lung, these studies have not been pursued much further.

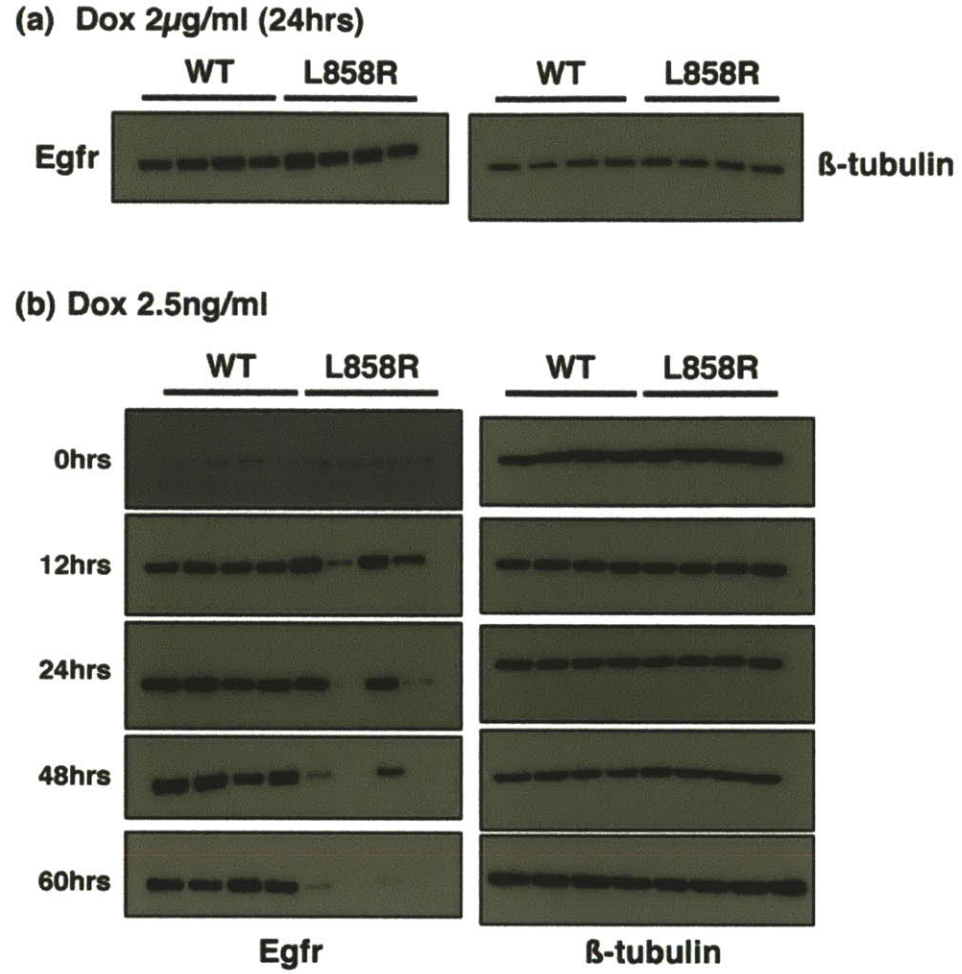
An inducible system to study mutant receptor downregulation

We hypothesized that our inability to identify the negative regulator of mutant Egfr might be connected to the constitutive expression of Egfr in both germline mutant MEFs and tissues, such that by the time any analysis is carried out a balance between mutant Egfr and negative feedback signaling has been achieved. In this scenario with Egfr maintained at low levels, the feedback protein is no longer required at levels significantly different from those in wild-type cells. We attempted to use Cre-mediated recombination, either in MEFs or tissues, to examine

the effects of acute mutant Egfr signaling, but encountered difficulties in achieving complete recombination in all cells within a suitable timeframe, together with the added complexity of moving from one to two alleles of expressed *Egfr*. We decided instead to find a system employing regulated ectopic expression of wild-type and mutant Egfr.

We settled on the FLP-in T-REx 293 system (Invitrogen) for this purpose. This system utilizes a line of HEK293 cells that contain a stably integrated CMV-TetR transgene, and a separate FRT site at a transcriptionally active genomic region. A doxycycline-regulated cDNA can be flipped into this locus, and compared with others targeted to the same location, eliminating confounding issues of the integration site of transgenes on expression and/or regulation. We anticipated that the doxycycline-inducible feature would allow us to examine the response to mutant Egfr signaling in a timescale of hours, rather than the days required for recombination and expression to occur in Cre/loxP systems. We cloned the wild-type and L858R mutant Egfr cDNAs into the pcDNA/FRT/TO expression vector and in combination with Flp recombinase flipped these cDNAs into the target FRT site, generating lines that are isogenic apart from a single nucleotide difference in Egfr. Individual clones were picked, expanded, and tested for Egfr induction. Approximately all clones expressed Egfr when treated with a high dose of doxycycline (2μg/ml) for 24hrs (Figure 4A), and four clones each of wild-type and L858R were characterized further.

We began by carrying out a time course of Egfr expression at a low dose of doxycycline (2.5ng/ml) in an effort to avoid possible issues associated with vast overexpression of the receptor. As shown in Figure 4B the system is relatively tight, and while both the wild-type and mutant receptor are expressed in response to doxycycline treatment, levels of the mutant receptor decrease dramatically between 24 and 48hrs post-induction. There is some variability between the mutant clones, and although the extent of downregulation varied, the overall trend was the same. We have not investigated this clone-to-clone variability further. We first


(a) Dox 2μg/ml (24hrs)
WT
L858R
WT
L858R
Egfr
ß-tubulin
(b) Dox 2.5ng/ml
WT
L858R
WT
L858R
0hrs
12hrs
24hrs
48hrs
60hrs
Egfr
ß-tubulin
(c)
(i)
WT
L858R
(ii)
WT
L858R
Egfr
ß-tubulin
48hrs
60hrs
WT
L858R
WT
L858R
Egfr
ß-tubulin
48hrs + Tarceva
60hrs + Tarceva
(d)
WT
L858R
Egfr
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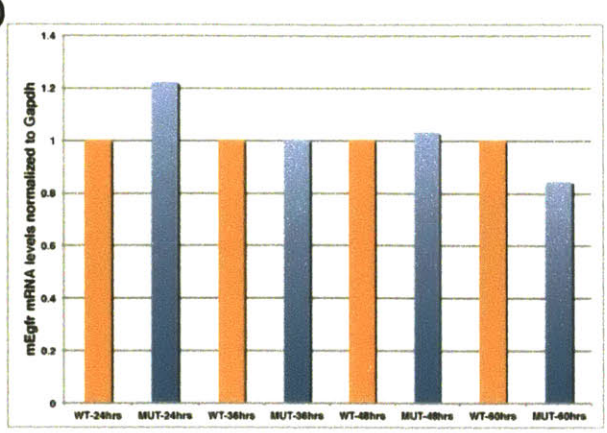
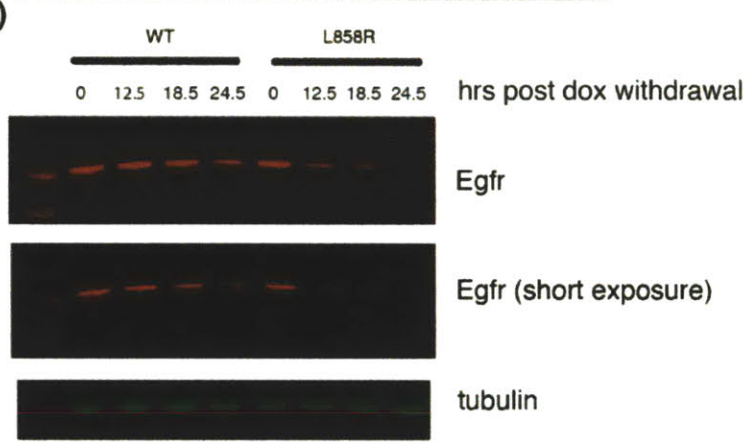
Egfr
ß-tubulin
10μm MG132
48hrs

(e)

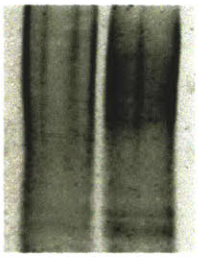

mEgfr mRNA levels normalized to Gapdh
1.4
1.2
1
0.8
0.6
0.4
0.2
0
WT-24hrs
MUT-24hrs
WT-36hrs
MUT-36hrs
WT-48hrs
MUT-48hrs
WT-60hrs
MUT-60hrs

(f)


WT
L858R
0 12.5 18.5 24.5 0 12.5 18.5 24.5
hrs post dox withdrawal
Egfr
Egfr (short exposure)
tubulin

(g)

(i)


IB: HA
IP: EGFR
WT L858R

(ii)

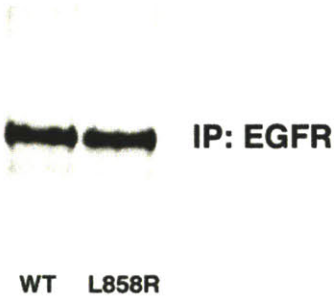

IP: EGFR
WT L858R

Figure 4. Recapitulation of mutant Egfr downregulation with an inducible system.

(a) Western blotting was used to test WT and L858R cells for Egfr induction using 2μg/ml doxycycline for 24hrs. **(b)** A time course using a low dose of doxycycline (2.5ng/ml) confirmed mutant receptor specific degradation by 48hrs as monitored by western blotting for Egfr. **(c)** Co-treatment of cells with tarceva and doxycycline diminishes mutant receptor degradation at both (i) 48hrs and (ii) 60hrs, implying activity dependent downregulation **(d)** MG132 treatment at 1μM and 10μM inhibits mutant receptor downregulation. **(e)** qPCR for Egfr mRNA levels in WT and L858R expressing cells at 24, 36, 48, and 60hrs post-induction. The comparison is made within each time point, with WT levels set to a value of 1 at each time point, and the relative expression of mutant Egfr determined. **(f)** Rapid degradation of mutant receptor upon doxycycline withdrawal. Egfr expression was induced for 24hrs and then doxycycline containing media was replaced by fresh media, and the decay in Egfr levels was monitored by western blotting. **(g)** The mutant receptor has increased levels of ubiquitination. WT and L858R cells were transfected with an HA-ubiquitin construct, and following induction Egfr was IP'd and the blots were probed with an HA-antibody.

investigated whether this mutant-specific downregulation was dependent on Egfr kinase activity. We compared mutant receptor levels at 48 and 60hrs, in the presence or absence of the TKI, tarceva, and found that induction in the presence of inhibitor was sufficient to counteract the downregulation (Figure 4C, i and ii). We confirmed a role for the proteasome in receptor downregulation by treating the cells with MG132, a broad acting proteasome inhibitor, which also resulted in mutant Egfr levels being restored at 48hrs (Figure 4D). Both of these results are reminiscent of those previously seen with the *Egfr* mutant MEFs (Chapter 2). Finally we confirmed that mRNA levels were approximately equivalent at all time points post-induction between the wild-type and mutant clones (Figure 4E).

To compare the stability of wild-type to mutant Egfr we cultured both sets of cells in doxycycline for 24hrs at which point the levels of expression are approximately equivalent. The doxycycline media was then exchanged for fresh media, halting Egfr transcription, with protein degradation accounting for all subsequent changes in Egfr expression. Egfr-WT protein was grossly stable for up to 18.5hrs post-doxycycline withdrawal, but levels of Egfr-L858R had already fallen precipitously by 12.5hrs (Figure 4F). To investigate whether the mutant receptor was undergoing differential ubiquitination we transfected a plasmid expressing HA-Ubiquitin into both sets of cells and induced receptor expression for 30hrs, followed by an IP for Egfr. A smear indicative of ubiquitination was detected only in the mutant receptor lane and not in the wild-type case (Figure 4G), coinciding with the proteasome inhibition and protein stability results. These data therefore suggest that, at least in the context of this 293 T-REx system, mutant Egfr is more unstable than wild-type, and that signaling from the receptor is responsible for its eventual ubiquitination and degradation.

A role for ERK pathway signaling in receptor degradation

We finally had a system where we could both monitor the acute effects of Egfr expression and recapitulate the downregulation, allowing us to functionally interrogate the degradation of the mutant receptor in a feasible timeframe. We had found that kinase activity was essential for this feedback and reasoned that the mutant receptor should be differentially phosphorylated, in tandem with the differential activation of associated downstream signaling pathways. We carried out a time course of induction, confirming the downregulation of mutant receptor by 48hrs (Figure 5A, i). Upon examining the kinetics of receptor phosphorylation we found that in contrast to the wild-type, the mutant receptor was highly phosphorylated at tyr-1068, while at tyr-1045 there appeared to be an early pulse in phosphorylation compared to a more graded response in the wild-type (Figure 5A, ii). We decided to focus on tyr-1068 due to the large differential in phosphorylation levels, and since it couples to the MAPK pathway through GRB2 recruitment we examined p-ERK levels over the time course. A pulse of p-ERK signaling at 8 and 16hrs, which coincided with the peak Egfr p-1068 signal, was found only in the mutant expressing cells.

To investigate whether ERK signaling was involved in mediating mutant EGFR degradation we utilized the MEK inhibitor, U0126. Since MEK acts directly upstream of ERK, an inhibitor of this kinase should, at least in theory, inhibit ERK activity. We took two mutant and two wild-type lines and carried out combination doxycycline/U0126 treatments as indicated (Figure 5B) with all cells receiving doxycycline for 48hrs, and either no U0126 (Group 1), 10μM U0126 for the final 34hrs (Group 2), or 10μM U0126 for the full 48hrs (Group 3). Although the suppression of MEK activity had no effect on wild-type levels, it led to an upregulation in mutant Egfr levels, but only when U0126 was present for the entire 48hr period (Figure 5B). The treatment of mutant lines with U0126 led to a paradoxical increase in p-ERK levels. This does not seem to be due to the U0126 no longer being functional as the p-ERK levels in wild-type clones remain reduced with 48hrs of treatment, and there is a buildup of p-MEK in both mutant

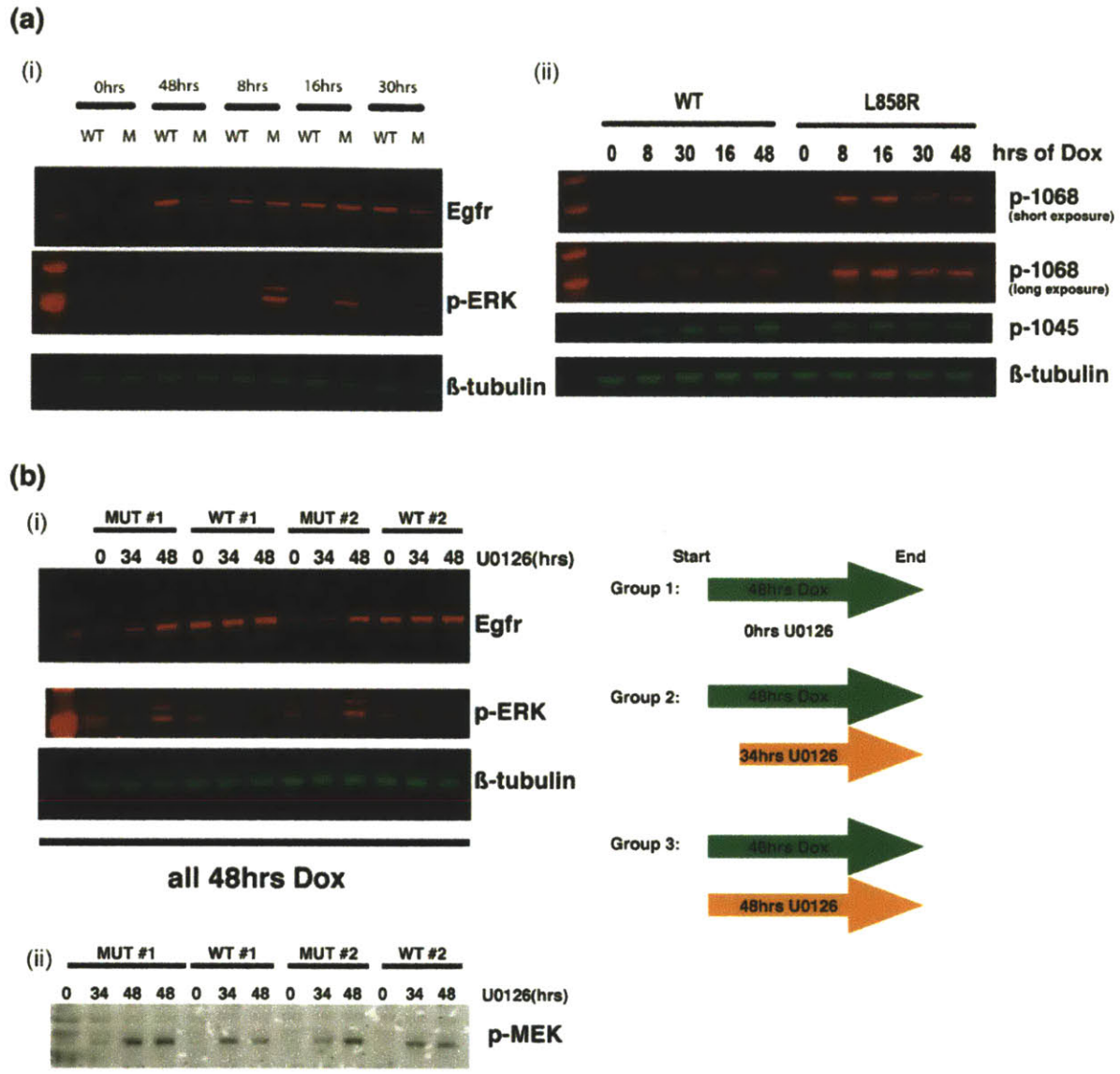


Figure 5. ERK pathway signaling is involved in mutant Egfr degradation.

(a) A doxycycline time course comparing WT and L858R expressing cells. Western blots of the time course examine (i) Egfr and p-ERK levels, and (ii) phosphorylation of Egfr. **Note**: the 16 and 30hr time points were loaded out of order in the WT set. **(b)** WT and L858R cells were divided into three groups and doxycycline and U0126 were administered in a time course as indicated. Western blotting revealed an increase in Egfr levels only in mutant cells co-treated with U0126 for 48hrs (Group3), along with an increase in p-ERK, and (ii) a build-up of p-MEK, a surrogate for U0126 activity.

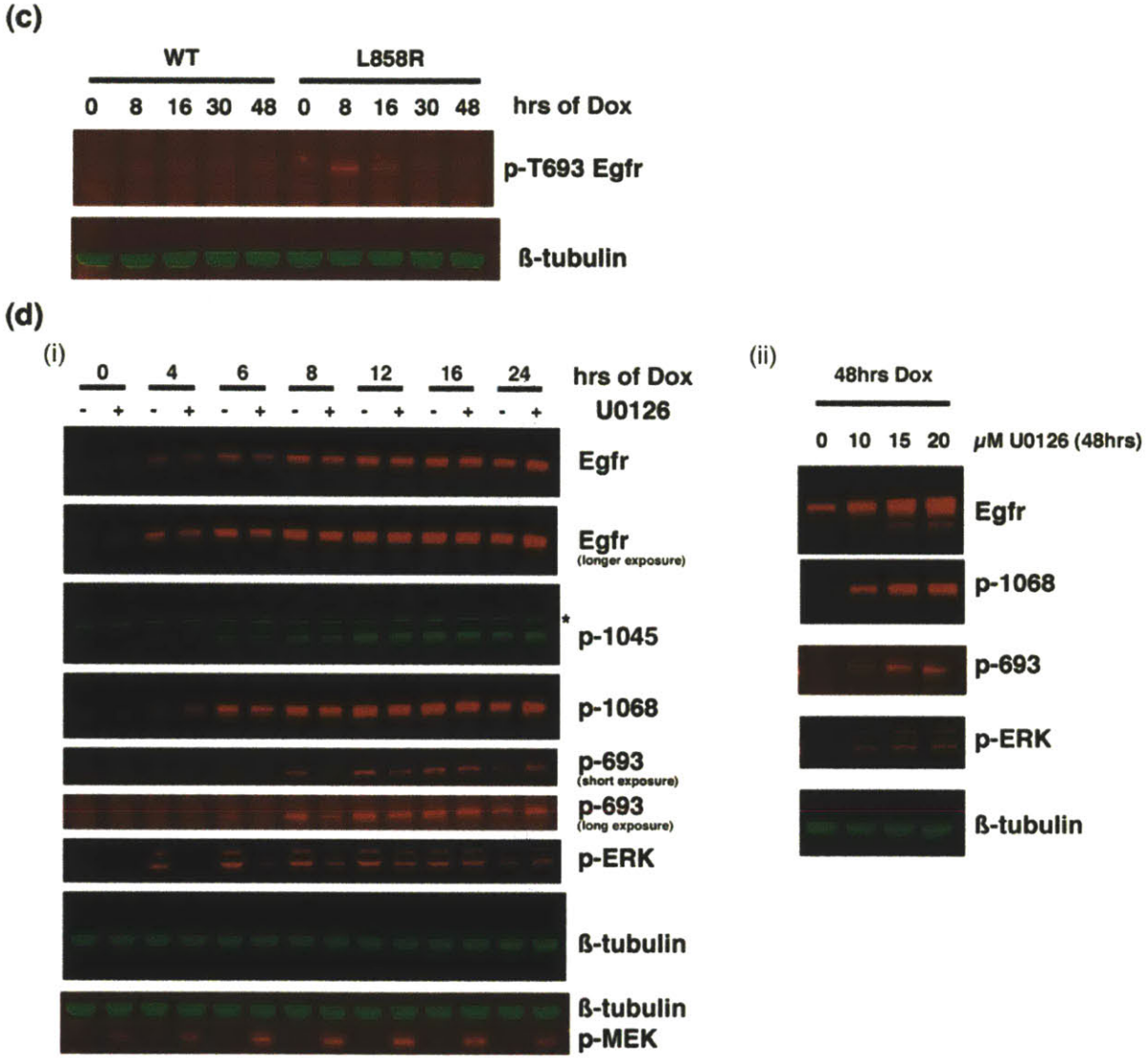


Figure 5 continued. ERK pathway signaling is involved in mutant Egfr degradation.

(c) By western blotting p-T693 Egfr is specifically upregulated in cells expressing mutant Egfr. **(d)** (i) A time course of doxycycline induction in the presence or absence of 10μM U0126 was carried out in L858R cells as indicated and pathway activation was monitored by western blotting. p-T693 Egfr is markedly reduced upon U0126 treatment and as before, p-MEK levels increase. * indicates a non-specific band in the p-1045 blot. (ii) The same L858R cells were treated with the indicated doses of U0126 for 48hrs and western blotting confirmed a dose-dependent upregulation in both Egfr levels and phosphorylation, and a more gradual increase in p-ERK levels.

and wild-type cells that is not detectable in the absence of inhibitor treatment (Figure 5B, ii). This suggests that the increase in p-ERK may be due to the upregulation in mutant Egfr levels and a coincident pulse of signaling through the MAPK pathway that simply overwhelms the ability of U0126 to maintain inhibition. From these data we conclude that ERK pathway signaling is involved in mutant receptor downregulation. On the basis of the inability of Group 2 conditions to rescue the downregulation, signaling in the initial 14-16hrs must at least partly contribute to this process.

ERK signaling modulates mutant Egfr phosphorylation

A connection between ERK pathway activation and EGFR phosphorylation at T693 (residue 669 in alternative numbering systems), a residue in the juxtamembrane domain, has been documented (Li et al., 2008; Takishima et al., 1991). This site is an ERK consensus phosphorylation site, and modification to alanine, such that it can no longer be phosphorylated, leads to an increase in global receptor phosphorylation, internalization, and ubiquitination; accordingly phosphorylation of T693 may play a role in maintaining the receptor at the surface and potentially resulted in increased signaling from the receptor. Given the universal property of a reduced internalization rate of the mutant receptor we investigated whether Thr693-Egfr was aberrantly phosphorylated in mutant expressing cells. In a time course of receptor induction, T693 was phosphorylated exclusively in mutant cells, and specifically at early time points, mirroring the dynamics of p-ERK in these cells (Figure 5C).

To examine the kinetics of p-T693 in mutant cells we carried out a 24hr time course in the presence or absence of U0126 (Figure 5D, i). Over the course of 24hrs any effect of U0126 on Egfr levels was not apparent, except for a slight increase at 24hrs, and phosphorylation of the canonical tyrosines, 1045 and 1068, was also unaffected. As expected if it is an ERK kinase substrate, phosphorylation of T693, was greatly reduced at 6 and 8hrs post-induction in the presences of U0126. At later stages the situation became more complex, as at 24hrs when the

levels of p-T693 were beginning to drop in the absence of U0126, p-T693 levels in cells cultured with the inhibitor had recovered from their initial lag and seem to be maintained. Upon examining p-ERK levels, we found that they are initially diminished in the presence of U0126, but subsequently increase and by 16hrs are restored to a level equivalent to that found in the untreated cells. p-MEK is normally undetectable, even in mutant cells which show a pulse of p-ERK, but in the presence of U0126 p-MEK builds up to very high levels, even when p-ERK activation seems to no longer be inhibited. We extended this time course out to 48hrs to confirm that mutant receptor levels do increase in the presence of U0126 in this particular experiment. We found that as we dialed up the amount of U0126 administered, Egfr protein levels increased in parallel, as did receptor phosphorylation at tyr-1068 and thr-693, while p-ERK levels increased above background but appeared to remain stable across differing doses of U0126. These results support a model that incorporates ERK signaling in mutant Egfr downregulation and highlight a poorly studied phosphorylation event on the receptor, p-T693, as a potential component of this process.

A role for phosphatases in Egfr regulation

Our previous analysis in MEFs and tissues from the germline mutant mice had been constrained by a lack of candidates known to act as negative regulators of the receptor and a similar situation presented itself with the 293 T-REx system. In conjunction with more targeted approaches we decided to also employ an unbiased method; hence we carried out expression profiling on wild-type and mutant cells, including three time points, 0hrs, 16hrs, and 30hrs. We included the uninduced controls for both lines as it was possible that there would be basal differences between clones that are independent of Egfr expression, and it was also essential to be able to discern what was actually induced. Our analysis of the array data (data not shown) revealed that the expression of wild-type Egfr resulted in no significant transcriptional changes at either 16 or 30 hrs post induction. This agrees with our observation of a general lack of

tyrosine phosphorylation of the wild-type receptor, since it represents the principal route by which downstream signaling pathways are activated. The induction of mutant receptor expression led to a pulse of expression changes at 16hrs, which although still above baseline, were greatly diminished in scale by 30hrs.

The kinetics of the expression changes mirror the timescale of downregulation and reinforce the necessity of an inducible system in which to study the underlying mechanism. Most of the changes identified from the microarray analysis converge on the ERK pathway, coinciding with the results of our western blot analysis. One of the categories significantly upregulated were phosphatases, specifically dual specificity phosphatases (DUSPs), including DUSP4 and DUSP6. DUSP4 has been previously found to be frequently lost in EGFR-mutant NSCLC (Chitale et al., 2009), and is a nuclear phosphatase for ERK, p38, and JNK, while DUSP6 is a cytoplasmic phosphatase with a high specificity for ERK1/2 (Owens and Keyse, 2007). We confirmed that there was modest upregulation of DUSP6 at the protein level specifically in mutant expressing cells at 8 and 16hrs (Figure 6A). Our earlier data with U0126 indicated that ERK signaling dynamics was playing a role in regulating Egfr expression levels, and since DUSP6 was a direct response to this ERK signaling we decided to investigate further what effect altering DUSP6 expression levels would have. We designed shRNAs against DUSP6 and tested their effect on DUSP6 protein levels over a time course of mutant receptor induction (Figure 6B). All three hairpins gave good knockdown, although when analyzed at the mRNA level sh3 was by far the most potent and knocked down both spliceforms of DUSP6 (data not shown). The relative importance of the spliceforms is unknown as no functional significance has been specifically attributed to either one.

We assessed the role of DUSP6 with our by now standard assay of blotting for total Egfr levels after 48hrs of doxycycline induction. We found that only DUSP6sh3 led to robust upregulation of mutant Egfr expression levels (Figure 6C), although when we extended the time course to 60hrs sh2 also upregulated mutant Egfr levels, even though at 48hrs it had only a

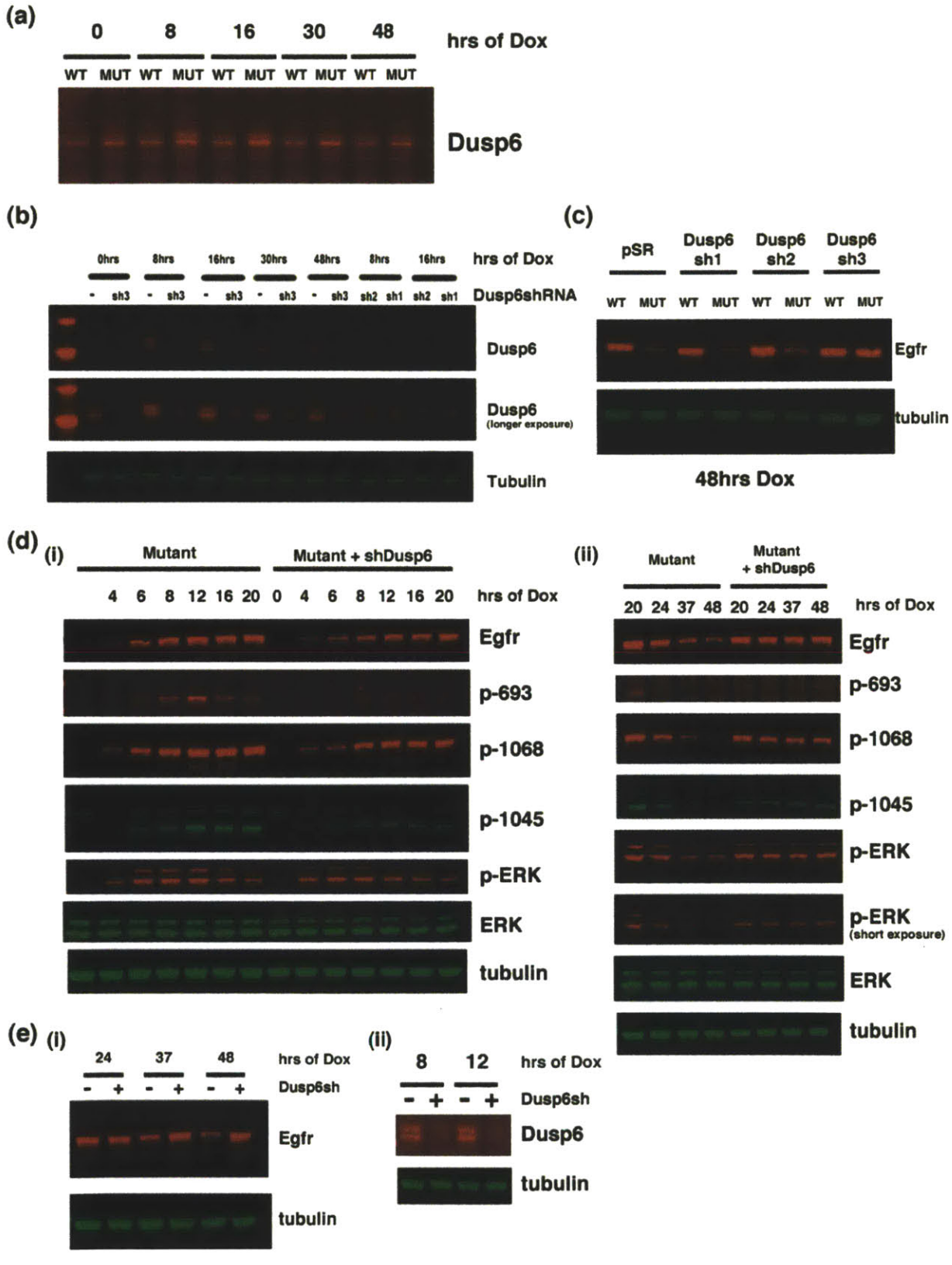

(a)
0
8
16
30
48
hrs of Dox
WT MUT WT MUT WT MUT WT MUT WT MUT
Dusp6
(b)
0hrs
8hrs
16hrs
30hrs
48hrs
8hrs
16hrs
hrs of Dox
- sh3 - sh3 - sh3 - sh3 - sh3 sh2 sh1 sh2 sh1
Dusp6shRNA
Dusp6
Dusp6
(longer exposure)
Tubulin
(c)
pSR
Dusp6 sh1
Dusp6 sh2
Dusp6 sh3
WT MUT WT MUT WT MUT WT MUT
Egfr
tubulin
48hrs Dox
(d)
(i)
Mutant
Mutant + shDusp6
4 6 8 12 16 20 0 4 6 8 12 16 20
hrs of Dox
Egfr
p-693
p-1068
p-1045
p-ERK
ERK
tubulin
(ii)
Mutant
Mutant + shDusp6
20 24 37 48 20 24 37 48
hrs of Dox
Egfr
p-693
p-1068
p-1045
p-ERK
p-ERK
(short exposure)
ERK
tubulin
(e)
(i)
24
37
48
hrs of Dox
- + - + - +
Dusp6sh
Egfr
tubulin
(ii)
8
12
hrs of Dox
- + - +
Dusp6sh
Dusp6
tubulin

Figure 6. Decreasing DUSP6 levels leads to an increase in mutant Egfr expression.

(a) Western blotting for DUSP6 in a doxycycline time course of WT and L858R cells shows a slight increase in DUSP6 levels in mutant cells at 8hrs and 16hrs. **(b)** Three shRNAs designed against DUSP6 were cloned into lentiviral vectors, and validated by blotting for DUSP6 over a time course in L858R cells infected with a virus expressing the indicated hairpin. **(c)** These three hairpins were stably expressed in WT and L858R cells and the effect of DUSP6 knockdown on mutant degradation was assessed by western blotting for Egfr levels at 48hrs post-induction. sh3 shows a full rescue, while sh2 gives a marginal upregulation in Egfr levels. **(d)** L858R cells with or without DUSP6sh3 underwent a doxycycline time course and Egfr levels and activity were monitored by western blotting. (i) The early time points revealed that p-T693 Egfr was almost completely prevented by the presence of the DUSP6 hairpin, while the later time points (ii) uncovered that knockdown of DUSP6 led to Egfr and ERK phosphorylation being maintained out to 48hrs. **(e)** Western blotting to confirm (i) DUSP6 knockdown leads to an increase in Egfr levels at 48hrs in the cells used in (d), and (ii) that the DUSP6 hairpin is acting on its target.

marginal effect. DUSP6sh3 was used for all subsequent analysis. As with U0126 treatment, we carried out a comprehensive time course to try to gain a more complete understanding as to how knockdown of DUSP6 might elicit an upregulation in Egfr levels (Figures 6D, i and ii). The most striking difference upon DUSP6 knockdown was the complete loss of p-T693-Egfr phosphorylation. This was accompanied by a marginal decrease in total Egfr levels, along with a reduction in p-1068 and p-1045. p-T693 has previously been linked with full activation of the receptor, and thus the loss of this phosphorylation event may have a knock-on effect on the global phosphotyrosine content of the receptor. The ERK phosphorylation dynamics show subtle differences in the context of DUSP6 knockdown, namely a more rapid induction, a reduction in peak expression, and along with tyrosine phosphorylation of the receptor, ERK phosphorylation is maintained at 48hrs. We confirmed that DUSP6sh3 did induce an upregulation in Egfr levels at 48hrs (Figure 6E, i) and that knockdown was appreciable (Figure 6E, ii). Our data suggest that DUSP6 knockdown leads to a general reduction in Egfr activity, and perhaps by avoiding the early pulse of activity, Egfr can avoid triggering downregulation and can maintain signaling. Although the dynamics require further investigation, together with our data on U0126 inhibition, it seems to suggest that downregulation may be initiated when Egfr signaling exceeds a certain threshold.

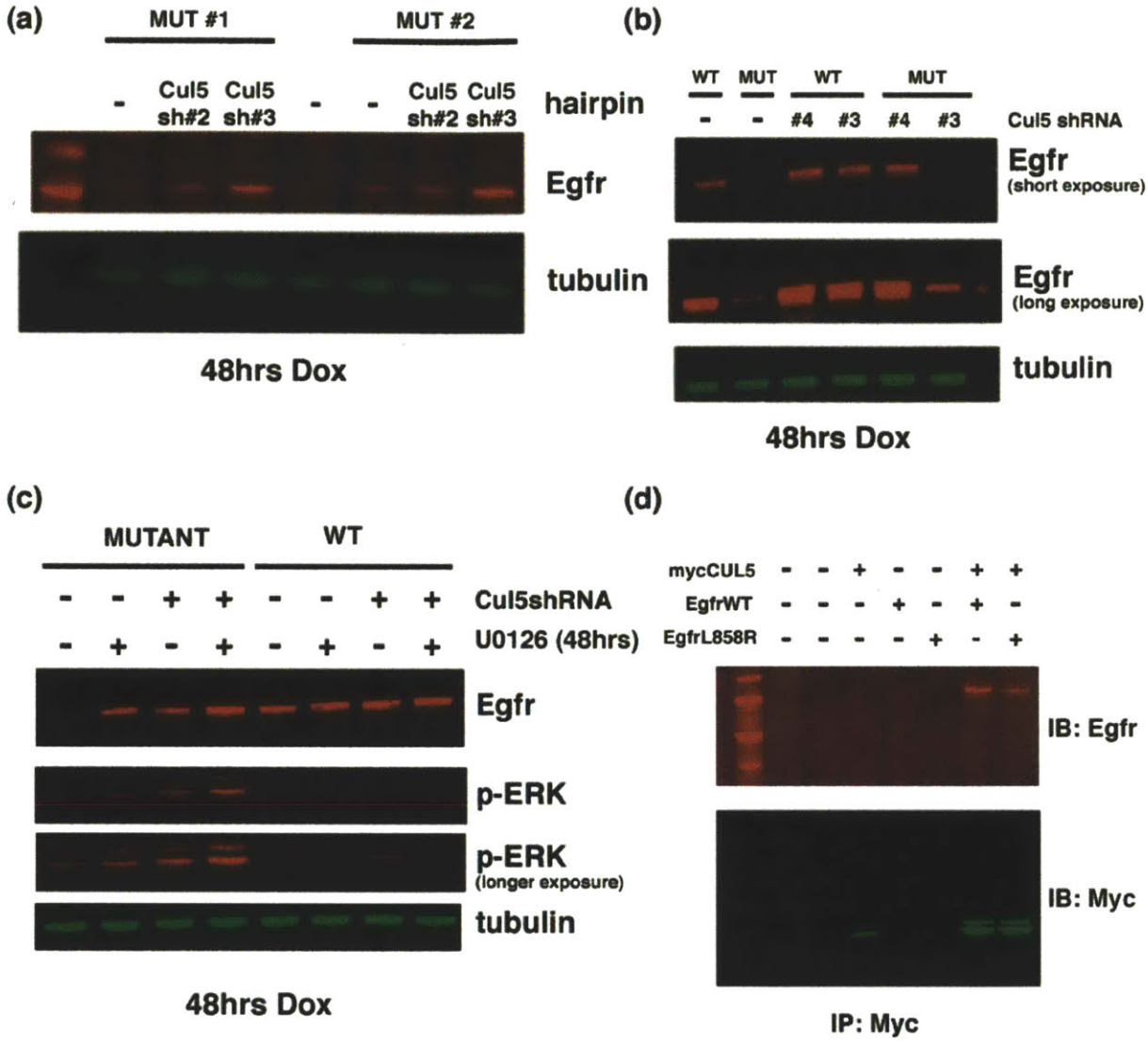
Identification of an E3 ligase that targets Egfr

The decreased stability of mutant Egfr in the 293 T-REx cells, the reversal of the downregulation upon proteasome inhibition (Figure 4D), together with the increased ubiquitination of the mutant receptor (Figure 4G) led us to conclude it would be prudent to attempt to identify the E3 ligase responsible for mutant receptor degradation. The identification of the E3 ligase would approach the mechanism of mutant Egfr downregulation from the reverse angle, and we hoped it would converge with the alternative approach of trying to connect the

signaling pathways immediately downstream of Egfr to decreased receptor levels, finally leading to a more complete understanding of the process of receptor downregulation.

The recent identification of CULLIN5 (CUL5) as an E3 ligase that acts primarily on hsp90 client kinases, including ERBB2 and HIF1-α (Ehrlich et al., 2009), led us to investigate whether mutant Egfr, itself an hsp90 client kinase, was also a target. We began by designing hairpins against CULLIN5, and compared mutant Egfr levels in two independent clones 48 hrs after doxycycline addition in the presence or absence of these hairpins (Figure 7A). One hairpin, Cul5sh3, resulted in an increase in mutant Egfr levels compared to controls. We subsequently found another hairpin that resulted in greater knockdown of CULLIN5, as assessed by qPCR (data not shown), and this led to a further increase in mutant Egfr levels, while having a marginal effect on wild-type Egfr expression (Figure 7B). Due to the similar end result of CUL5 knockdown or U0126 treatment on Egfr levels, we wanted to discern whether they were acting through the same, or independent pathways. We assayed Egfr expression in mutant and wild-type cells after 48hrs of doxycycline treatment, and also combined this with the presence of U0126, CUL5 knockdown, or both (Figure 7C). As expected wild-type Egfr levels were not affected either by U0126 treatment, CUL5 knockdown, or the combination. In contrast, mutant Egfr levels, and correspondingly p-ERK levels, were elevated in the mutant cells treated with either U0126 or CUL5 knockdown individually, and there seemed to be an additive effect when they were combined. Due to potential issues with either the inhibitor or the shRNA not being 100% effective this result is difficult to interpret, and all we can conclude is that both contribute to Egfr upregulation.

If CUL5 is directly regulating mutant Egfr levels they should be associated, even transiently, in a complex. Due to the relatively low expression levels of Egfr in the TReX system and the corresponding difficulty in IP'ing the receptor, we began by investigating whether we could co-immunoprecipate the receptor with CUL5 when both were ectopically expressed. We co-transfected both into 293FT cells and following an IP of tagged-CUL5 found that we could


(a)
MUT #1
MUT #2
- Cul5 sh#2 Cul5 sh#3 - - Cul5 sh#2 Cul5 sh#3
hairpin
Egfr
tubulin
48hrs Dox
(b)
WT MUT WT MUT
- - #4 #3 #4 #3
Cul5 shRNA
Egfr
(short exposure)
Egfr
(long exposure)
tubulin
48hrs Dox
(c)
MUTANT
WT
- - + + - - + + Cul5shRNA
- + - + - + - + U0126 (48hrs)
Egfr
p-ERK
p-ERK
(longer exposure)
tubulin
48hrs Dox
(d)
mycCUL5 - - + - - + +
EgfrWT - - - + - + -
EgfrL858R - - - - + - +
IB: Egfr
IB: Myc
IP: Myc

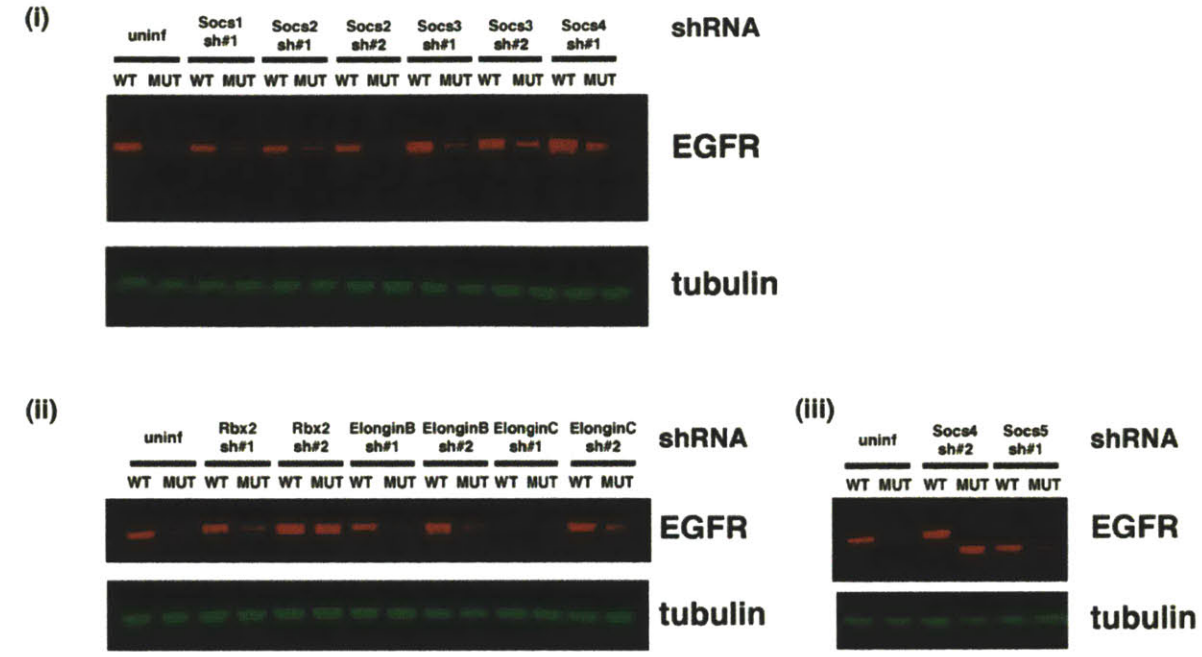


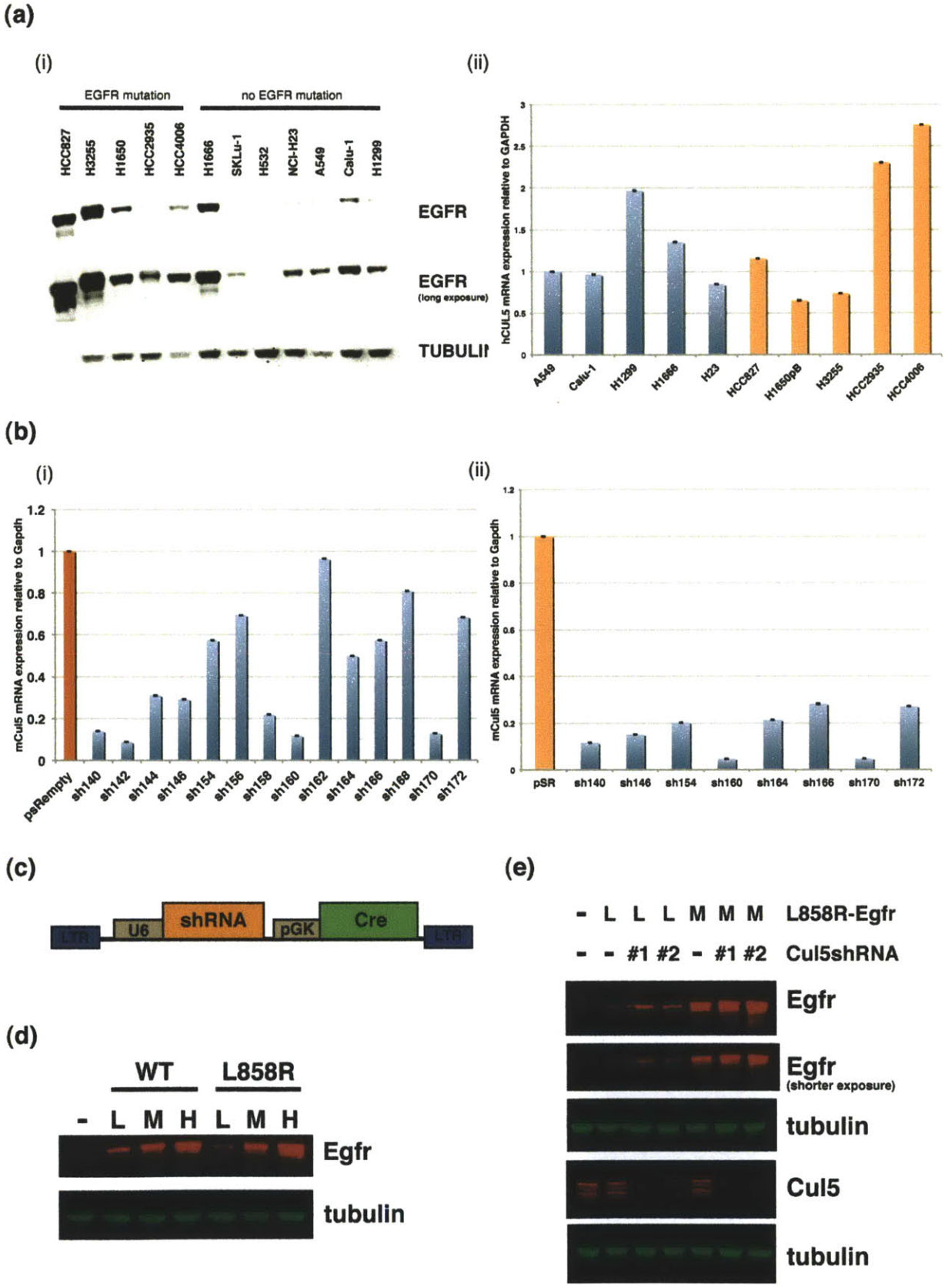
Figure 7. CULLIN5 is a candidate E3 ligase for mutant Egfr degradation.

(a) CULLIN5 knockdown in two independent mutant lines leads to upregulation of Egfr levels at 48hrs with one of the two hairpins tested. **(b)** A second hairpin that was more effective at knocking down CUL5 was identified, and it led to a further increase in Egfr levels at 48hrs post doxycycline induction. **(c)** The combination of CUL5 knockdown and U0126 treatment led to a further increase in mutant Egfr levels than either achieved alone, with a corresponding increase in p-ERK levels. **(d)** 293FT cells were transfected with the indicated plasmids and using an antibody against myc-tagged CUL5 both $Egfr^{WT}$ and $Egfr^{L858R}$ were found to co-immunoprecipitate with this E3 ligase. The prominent CUL5 doublet may represent a neddylated version of CUL5. **(e)** WT and L858R cells were infected with lentiviruses expressing shRNAs against all the putative components of the CUL5 complex listed and the effect on mutant Egfr levels 48hrs post doxycycline induction was monitored by western blotting. RBX2sh#2 and SOCS4sh#2 led to a detectable upregulation in mutant Egfr levels. Note: in **(e)**(iii) the gel ripped which is why the Egfr bands in the first three lanes appear to be migrating at a higher MW than the last three.

successfully bring down both the wild-type and mutant receptor (Figure 7D). In the context of a complex with the receptor CUL5 migrated as a doublet, which might be indicative of neddylation, a CUL5 modification essential to its E3 ligase activity. Cullins generally act as a scaffold and require a number of other proteins to be recruited for effective ubiquitination of their target proteins. If mutant Egfr was a veritable target of CUL5 we reasoned that the knockdown of other components of the CUL5 complex should also inhibit downregulation of the receptor. We designed hairpins against all of these potential components including RBX2, ELONGINB and ELONGINC, and SOCS1-5, which are typically involved in recruitment of the target protein. We investigated the effect of these individual hairpins in the typical 48hr doxycycline induction assay, and found that only hairpins targeting either RBX2 or SOCS4 led to an upregulation of mutant Egfr levels (Figure 7E). RBX2 is a RING finger protein known to complex with CUL5 and is essential for it's activity, while SOCS4 can associate with CUL5 amongst other cullins, but interestingly has been previously shown to interact with EGFR and promote it's degradation (Bullock et al., 2007; Gotoh, 2009; Kario et al., 2005).

Strategies to determine if CUL5 knockdown has any functional significance

If CUL5 is involved in regulating mutant EGFR levels in human tumors it is possible then that in those tumors that don't have EGFR amplification that CUL5 is lost, or indeed that CUL5 expression is decreased in EGFR mutant tumors in general when compared to tumors without EGFR mutation. In the absence of human tumor samples we decided to look for a connection between EGFR expression levels/amplification and CUL5 mRNA expression levels in NSCLC cell lines but there was no obvious correlation (Figure 8A). On the basis of the dramatic effect of CUL5 knockdown in mutant expressing T-REx cells, and the documented connection between CUL5 and other hsp90 client kinases, we decided to try to knockdown Cul5 in the lungs of conditional *Egfr* mutant mice. Due to the difficulties we have had in obtaining robust knockdown in the lung with a single integrant of a lentivirus we screened twenty hairpins to identify the most


(a)
(i)
EGFR mutation
no EGFR mutation
HCC827
H3255
H1650
HCC2935
HCC4006
H1666
SKLu-1
H532
NCI-H23
A549
Calu-1
H1299
EGFR
EGFR
(long exposure)
TUBULIN
(ii)
hCUL5 mRNA expression relative to GAPDH
A549
Calu-1
H1299
H1666
H23
HCC827
H1650pB
H3255
HCC2935
HCC4006
(b)
(i)
mCul5 mRNA expression relative to Gapdh
psRempty
sh140
sh142
sh144
sh146
sh154
sh156
sh158
sh160
sh162
sh164
sh166
sh168
sh170
sh172
(ii)
mCul5 mRNA expression relative to Gapdh
pSR
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sh146
sh154
sh160
sh164
sh166
sh170
sh172
(c)
LTR
U6
shRNA
pGK
Cre
LTR
(d)
WT
L858R
- L M H L M H
Egfr
tubulin
(e)
- L L L M M M L858R-Egfr
- - #1 #2 - #1 #2 Cul5shRNA
Egfr
Egfr
(shorter exposure)
tubulin
Cul5
tubulin

Figure 8. Functional experiments with CULLIN5.

(a) (i) Western blot for EGFR expression levels in a panel of EGFR mutant and non-mutant human NSCLC cell lines, and (ii) qPCR for CUL5 in the same lines. The expression level in A549 cells was arbitrarily set to one for comparative purposes. **(b)** Validating hairpins against mouse Cul5 in (i) *KP* lung cancer cell lines and (ii) *Egfr* mutant MEFs. sh140, sh160 and sh170 were used in all subsequent experiments. **(c)** Schematic of the lentiviral construct used to infect $Egfr^{LSL\text{-}L858R/+}$; $p53^{FL/FL}$ mice with shRNAs targeting Cul5. **(d)** Western blot of Egfr levels in NIH-3T3 cells expressing low (L), medium (M), or high (H) levels of either WT or L858R Egfr as a result of infection using different MOIs of an MSCV-hygro retrovirus containing either WT or L858R Egfr cDNA. **(e)** 3T3 cells expressing low and medium levels of L858R were re-infected with lentiviruses expressing either sh140 or sh160 against Cul5. After a pure population had been selected using puromycin, western blotting confirmed knockdown of Cul5 and showed a small increase in mutant Egfr levels in cells expressing a Cul5 hairpin.

potent version. At the time of analysis we did not have an antibody that detected either mouse or human CUL5 and so assayed knockdown at the mRNA level in both *KP* lung cancer cell lines (Figure 8B, i) and mutant *Egfr* MEFs (Figure 8B, ii). Three hairpins: sh140, sh160 and sh170, were identified as giving the most complete knockdown in both systems and were subsequently cloned into a lentiviral vector that we had designed that comprises a U6-shRNA-Pgk-Cre cassette (Figure 8C), and that had been used previously to achieve some degree of knockdown in the lung and robust knockdown in sarcomas (Young and Jacks, 2010). Cohorts of $Egfr^{LSL\text{-}L858R}$; $p53^{FL/FL}$ and $p53^{FL/FL}$ mice have been infected with either empty vector or vector expressing one of the Cul5 hairpins.

Due to the aforementioned issues with obtaining functional hairpins in the lung, we wanted to investigate whether Cul5 knockdown could elicit a phenotype in mutant Egfr expressing cells, in addition to increasing levels. We settled on assaying the effect of Cul5 knockdown in NIH-3T3 cells expressing a range of Egfr levels. To this end, we began by infecting NIH-3T3s with a retrovirus expressing either wild-type or mutant Egfr at different MOIs, to control the number of integrants. We selected pure populations and found that we had lines expressing low, medium and high levels of Egfr (Figure 8D). We proceeded to take the mutant Egfr lines and infect them with either empty vector or a lentiviral vector expressing one of the three hairpins known to effectively target mCul5. Pure populations were again selected, and the effect of a Cul5 hairpin on mutant Egfr expression levels assessed by western blotting. We found that Cul5 knockdown, which we could now verify with an antibody that recognizes mouse Cul5, led to a marginal increase in mutant receptor levels in both the low and medium Egfr expressing cells (Figure 8E). To assess the functional significance of this knockdown we took the original uninfected line, and each of the low, medium, and high mutant Egfr expressing lines, with or without each of the three Cul5 hairpins and plated the cells in soft agar in the presence or absence of mEgf. We decided on growth in soft agar as our functional readout for the ability of Cul5 to modulate the oncogenic potential of mutant Egfr as (i) it is the cell culture assay that

most accurately reflects the ability of cells to form a tumor *in vivo,* and (ii) mutant EGFR has previously been shown to form colonies in soft agar when overexpressed in NIH3T3 cells (Greulich et al., 2005). The results of this assay are shown in Figure 9. As the level of mutant receptor is increased there is a corresponding increase in the ability to form colonies in soft agar. Cullin5 knockdown positively modulates colony formation under all conditions, but has the most profound effect in cells expressing low levels of the mutant receptor. These results suggest that Cullin5 actively suppresses levels of mutant Egfr, principally when Cullin5 levels are in excess; if Egfr levels surpass a particular threshold Cullin5 becomes saturated, allowing the surplus receptor to eschew this mode of negative regulation. One prediction of this model is that deletion/mutation of CULLIN5, or associated factors, may occur specifically in EGFR mutant tumors devoid of amplification, and this warrants further investigation.

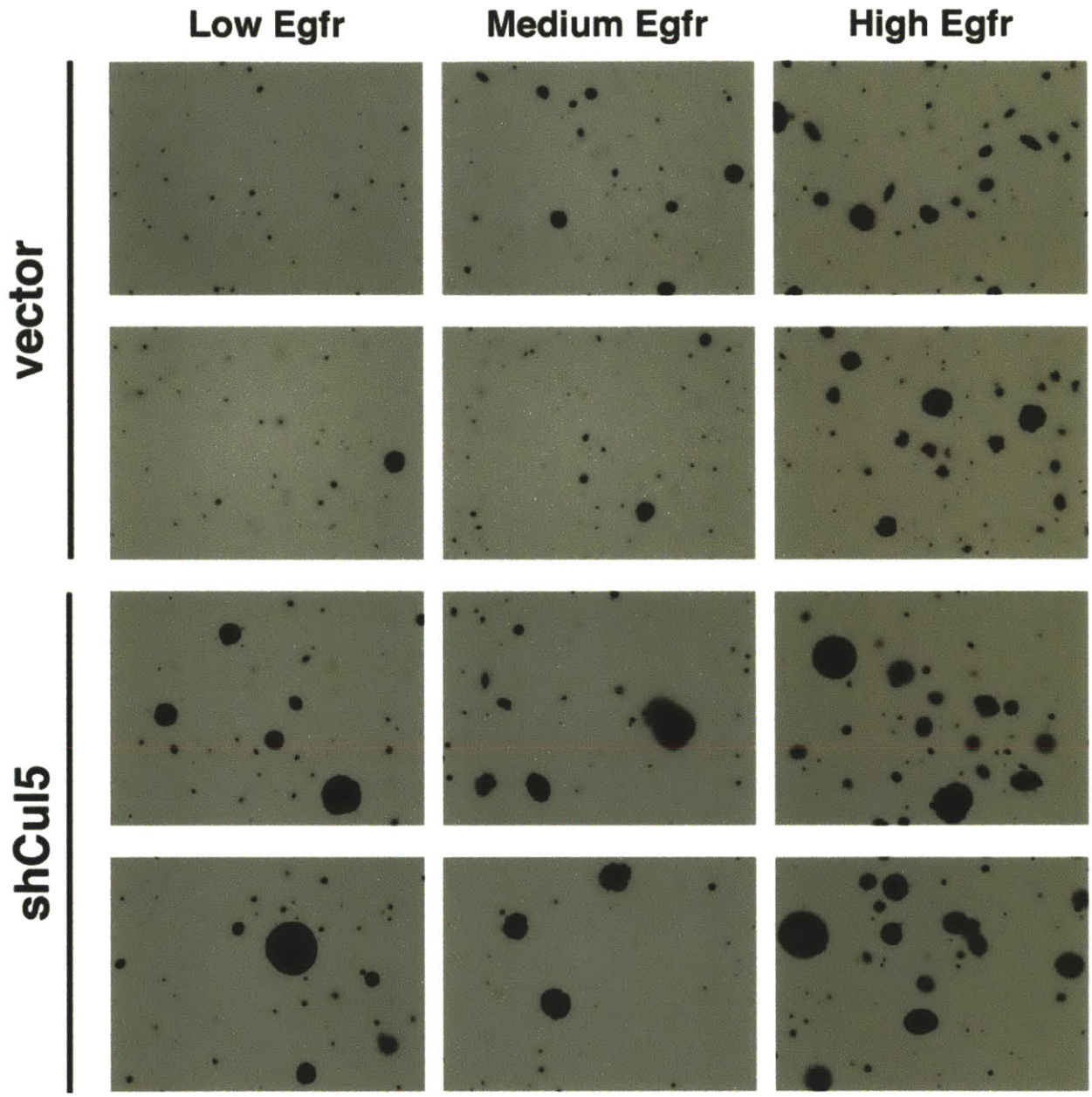

Figure 9. Cullin5 modulates mutant Egfr-mediated soft agar colony formation.

3T3 cells expressing low, medium or high levels of $Egfr^{L858R}$ were infected with empty vector or a hairpin targeting Cullin5. Cells were plated in soft agar and colony formation assessed after three weeks.

Discussion

The propensity of mutations in proto-oncogenes to initiate tumor formation depends on a delicate balance between exceeding homeostatic levels of growth promoting signaling while failing to engage, or disabling, tumor suppressor pathways that constrain oncogenic signaling and which may otherwise elicit oncogene-induced senescence (Braig et al., 2005; Collado et al., 2005; Young and Jacks, 2010). A third component of this model of tumor development comprises the negative feedback signaling modules and their role in regulating the levels of oncogenic signaling, both during tumor initiation and later progression. A spate of recent papers on the existence of the active suppression of signaling pathways by negative feedback elements in tumors and cell lines has promoted the idea that a balance of signaling is essential, even in established tumors (Chandarlapaty et al., 2011; Pratilas et al., 2009; Serra et al., 2011). Subtle differences in the dynamics or type of signaling elicited by distinct oncogenic proteins in a given signaling pathway may contribute to their relative induction of, or sensitivity to, feedback inhibition. This differential susceptibility to negative feedback constraints may result in the tumors evolving along different paths based on whether they need to disable or overcome this feedback.

The present study focused on the role of negative feedback in restricting the oncogenic potential of mutant Egfr, specifically in the initiation phase of tumor development. Although the number of documented EGFR feedback regulators is low, a handful of recent studies have focused on MIG-6, perhaps the most well characterized component of EGFR feedback inhibition. They have either elucidated a role for MIG-6 in restraining EGFR signaling, either under normal conditions or in response to chemically-induced papillomas (Ferby et al., 2006), found MIG-6 to be aberrantly phosphorylated in HBECs overexpressing mutant EGFR (Guha et al., 2008), or shown that loss of MIG-6 impacts EGFR trafficking in GBMs (Ying et al., 2010). However, they have relied upon established tumor lines, ectopic expression of EGFR, or deleted MIG-6 during development, a period when regulation of EGFR signaling is critical. We sought to examine

whether acutely adjusting levels of Mig-6 might disable the potent negative feedback acting on the mutant receptor. Deletion of Mig-6 failed to alter mutant Egfr levels but did result in alterations of the downstream signaling dynamics in MEFs in response to Egf. This suggests that in certain contexts Mig-6 might be specifically recruited to the mutant receptor to restrict the duration of signaling, and based on a recent study this could be a result of Mig-6 acting in concert with the SNARE protein, STX8, to shuttle EGFR into late endosomes (Ying et al., 2010), although this possibility has yet to be pursued.

Although this confirms a role for Mig-6 in the artificial environment of EGF-stimulated signaling of serum-starved cells, it was still unclear whether Mig-6 can exert a similar function under steady-state conditions, in the absence of acute growth factor stimulation. In an effort to understand the role of Mig-6 in Egfr regulation in the context of normal cellular homeostasis we induced the somatic loss of *Mig-6 in vivo.* In accordance with data from the knockout *Mig-6* mouse (Ferby et al., 2006; Zhang et al., 2005), the skin was the tissue most susceptible to Mig-6 loss, although in our case only the compound mutant $Egfr^{L858R}$; $Mig6^{-/-}$ mice developed papillomas in the timeframe of the study. This discrepancy could be due to strain background differences, somatic compared to germline deletion of *Mig-6*, or differences in the cell type in which the recombination occurred. These results confirm the increased, though actively suppressed, activity of the mutant receptor in the skin. In contrast, deletion of *Mig-6* in the lung failed to elicit a tumor phenotype. This result highlights the importance of context for feedback suppression of Egfr signaling, with Mig-6 appearing to play a more significant role in suppression of mutant Egfr signaling in the skin than the lung. However, due to the difficulty in assessing signaling in a small population of infected cells in the lung it is also formally possible that the loss of Mig-6 in the lung relieves feedback suppression of Egfr, but that unlike the skin, the signaling output still remains below the threshold required to elicit a tumor phenotype.
In contrast to the dramatic reduction in Egfr protein levels that had been observed in germline mutant mice, we could detect no corresponding alteration in downstream signaling in either

tissues or MEFs. We hypothesized that the initial expression of the mutant receptor resulted in a pulse of signaling, including the induction of a negative feedback loop, which over time led to a reduction in mutant Egfr levels as an effort by the cell to reduce signaling to a range similar to that of the wild-type receptor. Once lower levels of the mutant receptor were established there would be no need, or capacity, to maintain high levels of negative feedback signaling, and in the steady-state it would appear almost as if nothing, apart from receptor levels, was altered. While Cre-lox technology affords temporal and spatial control over the induction of oncogene expression, the timeframe between viral Cre addition, compete recombination, and protein expression is lengthy, and induction of expression is far from synchronous, making it difficult to look at signaling events in the hours immediately after oncogene induction. To circumvent this, we initiated studies in a doxycycline-regulated FLP-in system, in which expression of the gene of interest is induced within hours of doxycycline addition. At least qualitatively the induction is uniform, and by controlling the amount of doxycycline the level of the induced protein can also be modulated. Using this system we have begun to explore early signaling dynamics in response to either wild-type or mutant Egfr expression. Surprisingly the downregulation of mutant Egfr observed in tissues from the germline mutant mice was fully recapitulated using this system, occurring rapidly, within 48hrs of induction: a timescale not readily amenable to Cre-lox technology. This strong suppression of mutant Egfr levels reverts mutant signaling, with mutant expressing cells closely resembling wild-type expressing cells by 48-60hrs post-induction. A similar result had previously been seen when the oncogenic Jak2V617F was expressed in this system (Haan et al., 2009).

As discussed above, the components of Egfr feedback pathways are not well established and this system has uncovered a role both for ERK signaling and for CUL5, an E3 ligase associated with hsp90 client kinase degradation, in mutant Egfr degradation. The hyperactivation of the PI3K/AKT pathway in tumors and cell lines expressing mutant EGFR and the associated involvement of ERBB3 in mutant EGFR signaling (Engelman et al., 2005;

Sordella et al., 2004) has led to minimal investigation of the role of ERK pathway signaling in these tumors. A correlation has recently been found between the decreased internalization rate of the mutant receptor, reduced SHP2 phosphorylation, and lower levels of ERK signaling in mutant EGFR expressing cells, together with a role for ERK signaling in modulating gefitinib sensitivity (Lazzara et al., 2010). If ERK signaling is involved in mutant Egfr degradation this could explain why the ERK pathway is not hyperactivated in NSCLC cell lines harboring EGFR mutations. A fine balance between the growth promoting effects of ERK signaling and the induction of feedback that curtails receptor expression may be a critical component of mutant EGFR expressing cells, such that ERK signaling is maintained below a threshold that would induce high levels of feedback. The connection between ERK pathway activation and phosphorylation of Egfr at Thr-693, and the ablation of this phosphorylation upon DUSP6 knockdown in mutant expressing cells requires further investigation. Phosphorylation at this residue has been shown to reduce the internalization rate of the receptor and the contribution of this phospho-site to the reduced internalization rate of the mutant receptor observed in cell lines and MEFs should be determined.

One possible model that emerges from this work is that ERK pathway activation leads to phosphorylation of T-693 Egfr, resulting in the mutant receptor residing on the surface longer than is normal for an activated receptor thus making it a target for degradation by the E3 ligase CULLIN5. We have established roles for ERK signaling and CUL5 complexes in mutant receptor degradation, but currently lack evidence suggesting they act in the same pathway, or any clue as to what the underlying signals are which recruit CUL5 to the mutant receptor. Now that we have tools with which to modify Egfr degradation we should be able to get an improved understanding of whether the spatial distribution of the receptor is altered under these conditions, along with an improved view of ERK and DUSP-6 spatial dynamics in the course of mutant receptor expression. An alternative model to explain the downregulation observed is that the hyperactivated state of the mutant receptor simply leads to rapid internalization and

degradation. The phosphorylated state of the mutant receptor, as the major conduit to the activation of downstream signaling pathways, is no doubt involved in the degradation mechanism, but phosphorylation alone is likely insufficient to mediate this downregulation, as 1) the receptor is induced with minimal levels of doxycycline precisely to avoid supraphysiological levels of activated Egfr, 2) the scale of the transcriptional changes induced by expression of the mutant receptor are correspondingly low, indicative of low-level activation, and 3) in the case of either U0126 treatment or DUSP6 knockdown the receptor is highly phosphorylated at 48hrs but remains stably expressed.

EGFR mutant lung tumors fall into two broad categories, those with concurrent amplification and those without, with the latter representing the minority of cases. Our data suggest that mutant receptor activation leads to induction of negative feedback pathways, a safety mechanism employed by the cell to limit aberrant signaling under homeostatic conditions. There are two obvious routes to release the mutant receptor from the restrictions of this feedback control, (1) EGFR gene amplification to overwhelm the negative feedback components such that there is a sufficient pool of active receptor to induce oncogenic signaling, or (2) deletion or disabling of the negative feedback pathway. The tumors that evolve along these two very different paths may not be as similar as their response to TKIs would suggest, as alterations in EGFR levels can have both a qualitative and quantitative impact on signaling, while elimination of feedback mechanisms may have unanticipated pleiotropic effects on the signaling network. A deeper understanding of the feedback mechanisms involved in mutant EGFR regulation may have clinical implications, including the refined stratification of patient populations, but also more importantly the prospect of re-engaging or augmenting these feedback pathways in tumors with amplified EGFR may represent a viable therapeutic strategy.

Materials and Methods

RNA extraction, cDNA preparation and qPCR. RNA was isolated using either the Qiashredder and RNeasy Mini Kit (Qiagen) or with a standard Trizol extraction protocol. cDNA was generated using 1μg of RNA with the Superscript III kit (Invitrogen) and random hexamers. qPCR was carried out with Taqman probes using either an ABI PRISM 7000 Sequence Detection System Thermo Cycler (Applied Biosystems) or an ABI StepOne Plus System Thermo Cycler (Applied Biosystems). Taqman probes used were as follows: Egfr: Mm00433023_m1, Mig-6: Mm00505292_m1, Lrig-1: Mm00456116_m1, Gcf-2: Mm00521802_m1, AP2-α: Mm00495574_m1, Cullin5: Mm00512683_m1, Socs5: Mm01232423_m1, Spry2: Mm00442344_m1, Tbp: Mm00446973_m1, Gapdh: 4352932, CULLIN5: Hs00180143_m1, SOCS4: Hs00328404_s1, DUSP6: Hs00169257_m1, Hs00737962_m1, RBX2: Hs02621493_s1, GAPDH: 4352666.

shRNA design and cloning. All hairpin target sequences were designed using pSicoOligomaker v1.5 designed by A.Ventura (MSKCC), and the corresponding oligos were then cloned into either pSicoR-puro or the U6-shRNA-PgkCre previously designed in collaboration with M. Kumar and N. Young. All cloned hairpins were sequence verified. Hairpin sequences are available upon request.

Retrovirus preparation. Retrovirus was prepared by combining a 1:1 ratio of retroviral vector:pCL-Eco with TransIT-LT1 transfection reagent (MirusBio), followed by transfection of 293FT cells (Invitrogen). Viral supernatant was harvested and filtered at 48hrs and 72hrs post-transfection, and added directly to cells in combination with polybrene (10μg/ml). Selection was initiated 24hrs after the final addition of virus, and cells were maintained in selection for 48-72hrs for puromycin (2μg/ml) (Sigma) or 7-10 days for hygromycin (100-400μg/ml) (Sigma), or until all uninfected cells were killed.

EGF internalization assays. EGF internalization assays were carried out by M. Lazzara as previously described (Lazzara et al., 2010).

Lentivirus preparation and infection. Lentivirus was prepared as previously described (Rubinson et al., 2003). Briefly, 293FT cells were plated and 24hrs later when the cells were at 50% confluency they were transfected with a 4:3:1 mix of vector:delta8.2:VSVg combined with TransIT-LT1 transfection reagent (MirusBio). 48hrs and 72hrs post-transfection viral supernatant was harvested and filtered. For cell-culture experiments 6 well dishes were used and viral supernatant was added directly to cells plated in 6 well dishes in combination with polybrene (10μg/ml), and puromycin selection (2μg/ml) was initiated 24hrs after the final addition of virus. For *in vivo* experiments virus was prepared using 15cm dishes of 293FT cells and the 48hr and 72hr supernatants were pooled, and ultracentrifuged for 2hrs, at 4°C and 25,000 rpm. The viral pellet from each 15cm plate was resuspended in 200ul of 1X HBSS, pH 7.4 and tittered for Cre expression as previously described (DuPage et al., 2009). Virus that was to be administered within 4-5 days was stored at 4°C, otherwise it was aliquoted and stored at -80°C until needed.

Protein extraction and immunoblots. Cells were always lysed on ice following two washes with ice-cold PBS. The extraction buffer used was as follows: 10% Triton, 50mM $Tris_{7.4}$, 150mM NaCl, 0.5% sodium-deoxycholate, 0.1% SDS, 1mM DTT, 1mM EDTA, supplemented with Mini-protease inhibitor tablets (Roche) and either Phos-stop (Roche) or phosphatase inhibitor cocktails 1 and 2 (Sigma). Lysates were rocked at 4°C for 15mins followed by a 14,000rpm spin, 15mins, 4°C. Supernatants were quantitated using the BCA assay (Pierce), and diluted with a standard sample buffer containing β-mercaptoethanol. Lysates were run on SDS-PAGE gels, made in house or purchased from Invitrogen, and transferred to either PVDF or Nitrocellulose in the case of all Licor blots. Antibodies used were: EGFR (sc-03, Santa Cruz, Labvision, Ab-17), Mig-6 (Sigma, R3028(for mouse Mig6), R2903(for human MIG6)), LRIG1 (Abcam, ab36707),

GCF2 (BD, 612160), AP2-α (Santacruz, sc-184), β-tubulin (Sigma T4026, CST #2128), α-tubulin (CST #3873), Sprouty2 (Abcam), HA (Covance, MMS-101P), p-ERK (CST #4370), ERK (CST #9107), EGFRp-1068 (CST #3777), EGFRp-1045 (Abcam, ab24928), p-MEK (CST #9121), hsp90 (CST #4877), EGFRp-T693 (Abcam, ab52186), Dusp6 (Abcam, ab76310), Cullin5 (Bethyl labs, A302-173A), anti-myc 9E10 (Santacruz, sc-40). Blots were processed with either HRP-conjugated secondary antibodies and ECL+ reagents (Amersham), or with Licor IR secondary antibodies and imaged on a Licor machine. For Egf stimulation experiments MEFs were serum-starved for 18hrs prior to stimulation with 100ng/ml mEgf (Sigma), and all protein extractions were done on ice.

Preparation of stable T-REx lines. Stable T-REx lines were prepared as directed by the supplier (Invitrogen). Wild-type and mutant murine Egfr cDNA was cloned into the pcDNA/FRT/TO vector and sequence verified. 293 T-REx cells were maintained in 15μg/ml blasticidin and 100μg/ml zeocin until the day of transfection. The pcDNA/FRT/TO-Egfr vectors were mixed with pOG44 in a 1:18 ratio and transfected into 293 T-REx cells at 75% confluency using MirusLT1. 24hrs later the transfection mix was removed, and a further 24hrs later the cells were split 1:10 and selection was initiated using 125-150μg/ml hygromycin and 15μg/ml blasticidin for 24-48hrs, followed by 100μg/ml hygromycin and 15 μg/ml blasticidin for 10 days. Individual clones were then picked, expanded and tested for loss of lacZ expression, zeocin sensitivity, and doxycycline-inducible Egfr expression by western blot. Only clones that satisfied all three criteria were used in further studies and clones were never pooled.

Immunoprecipitation. Cells were transfected with HA-Ubiquitin using Lipofectamine 2000 (Invitrogen). Cells were induced with doxycycline for 30hrs with MG132 added for the final four hours. Cells were scraped in cold PBS, and pelleted at 1000rpm, at 4°C. Cells were then lysed in NP-40 lysis buffer for 15mins while rocking at 4°C, then insoluble material was pelleted with a 15min spin at 14,000rpm at 4°C. Protein lysates were quantitated with a BCA assay (Pierce),

and 1.5mg of protein was used for the IP. Lysates were pre-cleared for 1hr with Sepharose-6B beads (Sigma) and then incubated with 5µl of sc-03 antibody and 10µl each of Sepharose 6B beads and protein G Sepharose (Amersham, 17-0618-01) and rotated overnight at 4°C. Beads were pelleted and washed four times with lysis buffer and then resuspended in sample buffer and boiled before loading on a 6% SDS-PAGE gel.

Doxycycline time courses. Time course experiments involving the 293 T-REx cells were carried out in one of two ways. For some of the initial experiments and the time courses used in the array experiments all cells were harvested at the same time, with the media changed on cells 12hrs prior to the addition of doxycycline. This was done to avoid any issues associated with the fresh addition of serum. For all other time courses the media was changed upon doxycycline addition and again all time points were harvested together. A fresh stock of doxycycline (Sigma) was made every 8-12 weeks and kept at -20C.

Cell culture. MEFs were generated from E13.5 embryos and cultured in DMEM (DME, 10% FBS, 2mM glutamine, 1X Pen/Strep). 293FT cells and T-REx cells were also cultured in DMEM, but the latter was always cultured with tet-FBS (Clontech). Human NSCLC cell lines were cultured in either DMEM or RPMI as specified by ATCC, or in ACL-4 (H3255). H3255 cells were a kind gift from P. Janne. MG132 was purchased from EMD Biosciences. Tarceva was either purchased from Selleck Chemicals or a stock was also obtained from Genentech, and resuspended in DMSO. U0126 was purchased from CST, resuspended in DMSO, and aliquots stored at -20C.

Soft-agar assays. A 1:1 mix of 1.6% agarose (Seaplaque) and 2X media (Invitrogen) was made and 1.5ml was added to each 35mm dish and allowed to set at RT for 30mins. A 1:2:1 mix of 2X media: 1X media: 1.6% agarose was made and aliquoted with an appropriate number of cells, and where appropriate Egf to a final concentration of 20ng/ml was added. The volume

of cells and ligand added were removed from the volume of 1X media to preserve the correct total volume. 1ml of this mixture was then added to each plate and they were left to set for 1hr at RT. Plates were incubated at 37°C for 3 weeks, and a few drops of media, containing Egf where appropriate, were added to the plates each week to prevent them from drying out.

Mice. *Mig-6*$^{FL/FL}$ mice were kindly provided by F. DeMayo (Baylor College of Medicine, Houston, TX), and *Trp53*$^{FL/FL}$ mice were provided by A. Berns (Netherlands Cancer Institute). Egfr^{L858R}, Egfr$^{LSL\text{-}L858R}$ and R26^{CreER} strains were generated in the Jacks lab. Adenoviral and lentiviral intratracheal infections was carried out as previously described (DuPage et al., 2009). For experiments utilizing the CreER allele, tamoxifen (Sigma) was resuspended at 10mg/ml in corn-oil (Sigma) and four doses of 4.5mg/40g body weight were administered intraperitoneally every other day. Tissues were fixed in 10% formalin overnight, and processed as previously described (Jackson et al., 2001). Animal studies were approved by the Massachusetts Institute of Technology's Committee for Animal Care and conducted in compliance with Animal Welfare Act Regulations and other federal statutes relating to animals and experiments involving animals, and adheres to the principles set forth in the 1996 National Research Council Guide for Care and Use of Laboratory animals (institutional animal welfare assurance number, A-3125-01).

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Chapter 4.

Modeling EGFR^{-L858R} gene amplification in the mouse

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Keara Lane performed all experiments in the laboratory of Tyler Jacks

Abstract

The amplification of oncogenes is a recurring feature of many human tumors, but the contribution of gene amplification to particular stages of tumor development, or the molecular requirements for amplification to occur are unknown. An emerging question in the cancer biology field is whether tumors that develop with high-level expression of a mutant oncogene from the outset are similar to those that progress from a single mutant copy to amplification over a more protracted time period, and whether the cellular signaling circuitry is differentially rewired in each case. EGFR is mutated and coincidentally amplified in NSCLC, but the relative contribution of mutation and amplification both to tumor phenotype and therapeutic sensitivity is not clear. Mutation and immunohistochemistry (IHC) data from human tumor samples supports a model whereby mutation precedes amplification. Using a yeast endonuclease, I-Sce1, and an allele that contains target sites for this enzyme engineered telomeric to mutant *Egfr* on chromosome 11, we attempted to initiate breakage-fusion-bridge (BFB) cycles in the lung, as these are thought to be a precursor to gene amplification. Our inability to elicit tumor formation using this strategy highlights the limitations in our understanding of how amplicons form in human tumors or the particular context required. While it would provide tremendous insight into mutant EGFR tumor development, a model of targeted gene amplification has eluded us, and remains one of the significant challenges facing the mouse modeling community.

Introduction

One of the original hallmarks of cancer is genomic instability (Hanahan and Weinberg, 2000). Depending on the tumor type this can be manifest as gains or losses of whole chromosome arms, translocations, or focal amplification and deletions. Amplification of proto-oncogenes and their requisite overexpression is proposed to play a driving role in tumor evolution, and a comprehensive analysis of cancer genomes would necessarily help identify these key players, many of which may represent therapeutic targets. The advent of high resolution SNP analysis has allowed genomic alterations in human tumors to be characterized on a genome-wide scale (Beroukhim et al., 2010). A global analysis of copy number changes in lung adenocarcinoma found large-scale gains to be more frequent than focal amplifications, but 24 recurrent regions of focal amplification were identified, of which only 10 contain known proto-oncogenes, of which *EGFR* is one (Weir et al., 2007).

Oncogenic lesions typically take the form of either a mutation resulting in constitutive activity, or overexpression manifested through increased transcription, decreased turnover, or more typically through gene amplification, leading to qualitative and quantitative changes in signaling. The canonical oncogenes, *EGFR, ERBB2, MYC* and *RAS* are all amplified in human tumors, and *ERBB2* amplification is the quintessential case of an amplified gene being the diagnostic criteria for administration of a targeted therapy, Herceptin (Mass et al., 2005). The continued identification of amplified genes contributes to our global characterization of human cancer genomes, but there is a dearth of understanding as to either the basic conditions required for gene amplification or the initiating event(s) responsible for this lesion. In contrast to other genetic events that occur in tumors, the specific contribution of gene amplification to tumor initiation, progression, or therapeutic response is poorly understood, and is coupled with the corresponding absence of a definitive model as to how this fundamental oncogenic lesion occurs. This is primarily a reflection of our current inability to dissect this lesion in anything but the end state.

Gene amplification has been documented exclusively in a retrospective manner in human tumor samples. BFB cycles are posited as a potential underlying mechanism based on the observation of associated BFB features such as anaphase bridges, inverted repeat structures, and common breakpoint regions in many tumor amplicons (Gisselsson et al., 2000; Hellman et al., 2002). The use of model systems to explore the process of proto-oncogene amplification initially focused on the amplification associated with resistance to drugs, such as methotrexate and PALA, in cultured cells. These studies have provided evidence that p53-mediated cell cycle arrest in response to catastrophic DSBs acts a limiting factor in amplification, and have correlated expression of common fragile sites (CFSs) with the initiation of BFB cycles, at least in the context of this cell culture model of drug-induced amplification (Coquelle et al., 1997; Livingstone et al., 1992; Windle et al., 1991; Yin et al., 1992). The relative contribution of the double strand break lesion, the genomic context in which CFSs are found, or other unrelated factors, to the initiation of amplification is unknown, and has been tackled primarily through work in cell culture. CHO cells with a restriction site for the yeast endonuclease, I-Sce1, integrated telomeric to *DHFR* underwent methotrexate-induced *DHFR* amplification at a higher frequency upon I-Sce1 expression (Pipiras et al., 1998). Mouse ES cells generated with an HSV-tk marker gene and containing a centromeric site for I-Sce1 integrated in subtelomeric regions were subjected to I-Sce1 and ganciclovir and found to initially contain inverted repeats, followed by amplification of the region, and finally the process was terminated by the addition of telomeric sequences (Lo et al., 2002). While all of this implicates DSBs as the initiating lesion, formal evidence is lacking.

DSBs occur normally in cells, either through replication fork stalling or collapse, exposure of DNA to mutagens, irradiation, or other genomic insults. DSBs are tolerated in lymphocytes during V(D)J recombination, but in any other context they represent a potentially catastrophic lesion, and as such DNA repair mechanisms, including non-homologous end joining (NHEJ) or homologous recombination (HR), are essential for their resolution and to avoid

apoptosis. DNA repair processes are also essential to counteract many forms of genomic instability and per se restrict tumor formation. A series of mouse models based on deficiency of NHEJ factors in conjunction with loss of *p53* were found to elicit tumor phenotypes comprising focal amplifications (Sharpless et al., 2001; Yan et al., 2006; Zhu et al., 2002). *p53* deficiency appears to be an essential component of the amplification process, as it creates an environment where DSBs are tolerated and persist, and cell cycle checkpoints fail to be activated; thus increasing the chances of aberrant entry into S-phase, sister chromatid fusion, anaphase bridge formation, and the beginnings of the BFB cycle. While the lymphomas that developed in the $p53^{-/-}$; $Xrcc4^{-/-}$ mice were dependent on Rag-induced breaks, the exact contribution of DSBs to the other amplifications identified remains undetermined.

To attempt to establish a system that would allow us to induce gene amplification in murine cells in a targeted manner we developed a strategy inspired by a similar approach that utilized I-Sce1 to increase the rate of *DHFR* amplification upon methotrexate administration (Pipiras et al., 1998), albeit with some major modifications. NSCLC patients with *EGFR* mutations often have concurrent gene amplification, but the contribution of amplification to tumorigenesis is unknown (Nomura et al., 2007; Takano et al., 2005). Based on the work outlined in chapters 2 and 3 we hypothesized that our mutant *Egfr* allele was below the critical threshold needed for tumor formation, but that amplification of the allele might be sufficient to overcome that, and as such we used this allele as our 'amplification-prone' background on which to carry out our studies. Our strategy was based on evidence that a DSB may be the required initiating lesion for ensuing BFB cycles, and that deficiency in both p53 and DNA repair mechanisms should increase the probability of amplification. We constructed an allele telomeric to $Egfr^{LSL\text{-}L858R}$ that contained I-Sce1 sites to act as a targeted DSB, and subsequently through a number of different approaches expressed the endonuclease in target cells and monitored for tumor formation.

On the basis of our proposed strategy to induce amplification, as outlined in Figure 1, we decided on four conditions that had to be achieved for the system to work: 1) I-Sce1 had to be expressed at high enough levels to induce DSBs with the required efficacy, 2) the targeted cells expressing I-Sce1 had to be actively cycling to allow DSBs to either occur, or be present, in S-phase, 3) the efficiency of cellular DNA repair mechanisms had to be impaired to allow sufficient opportunity for sister chromatid fusion, and also to prevent NHEJ-based repair from repairing the site as this would eliminate a number of bases and prevent additional breaks, and 4) a phenotype had to manifest upon modest mutant Egfr overexpression. Meeting each of these criteria proved to be an unexpected challenge, and our inability to elicit tumors with this approach suggest that there are a number of technical hurdles that need to be overcome before a mouse model of gene amplification can be engineered. Furthermore, this provides evidence that perhaps the context of the DSB, whether in location, cell-type, or timing of induction, is important if a DSB is to result in ensuing amplification.

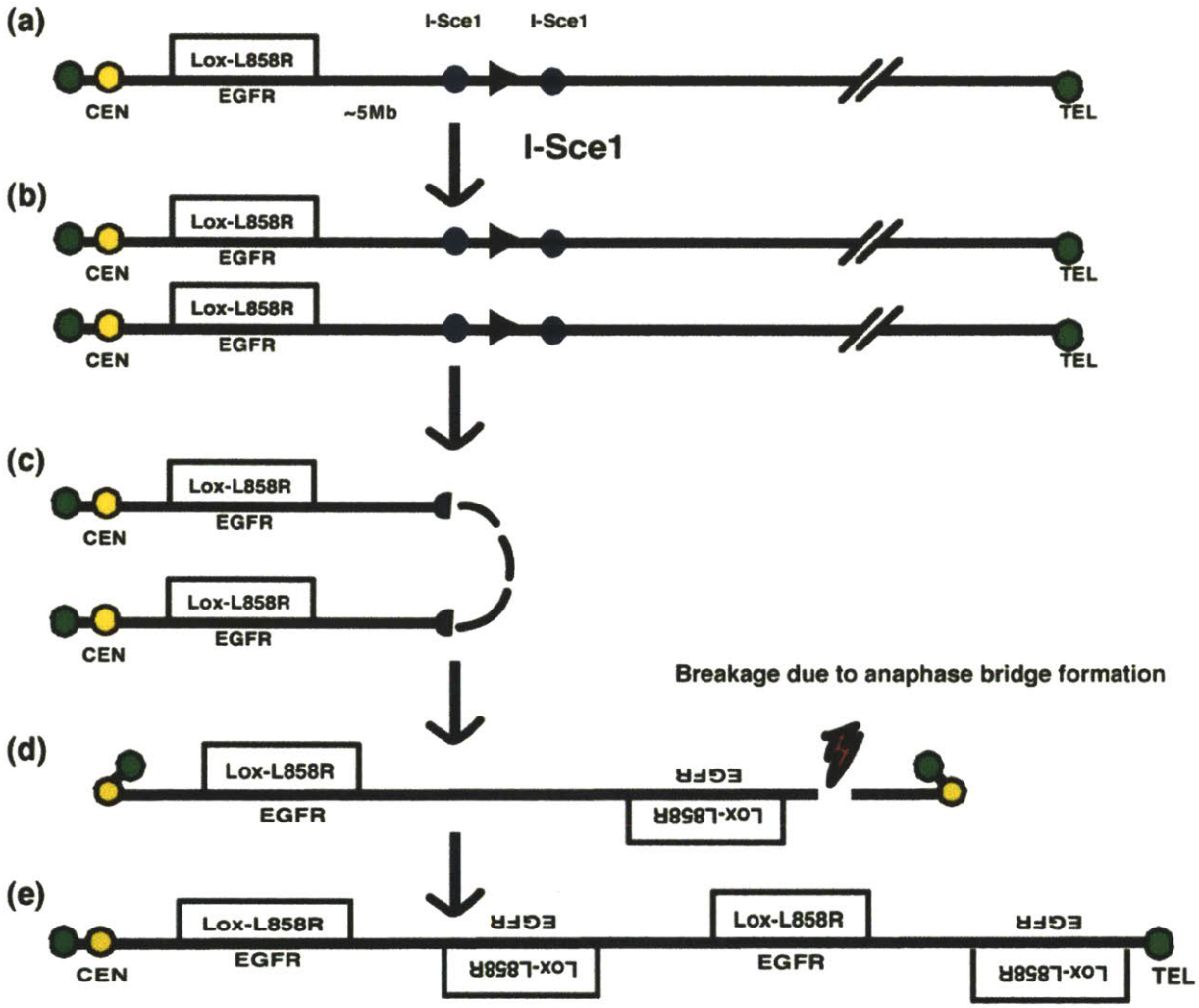


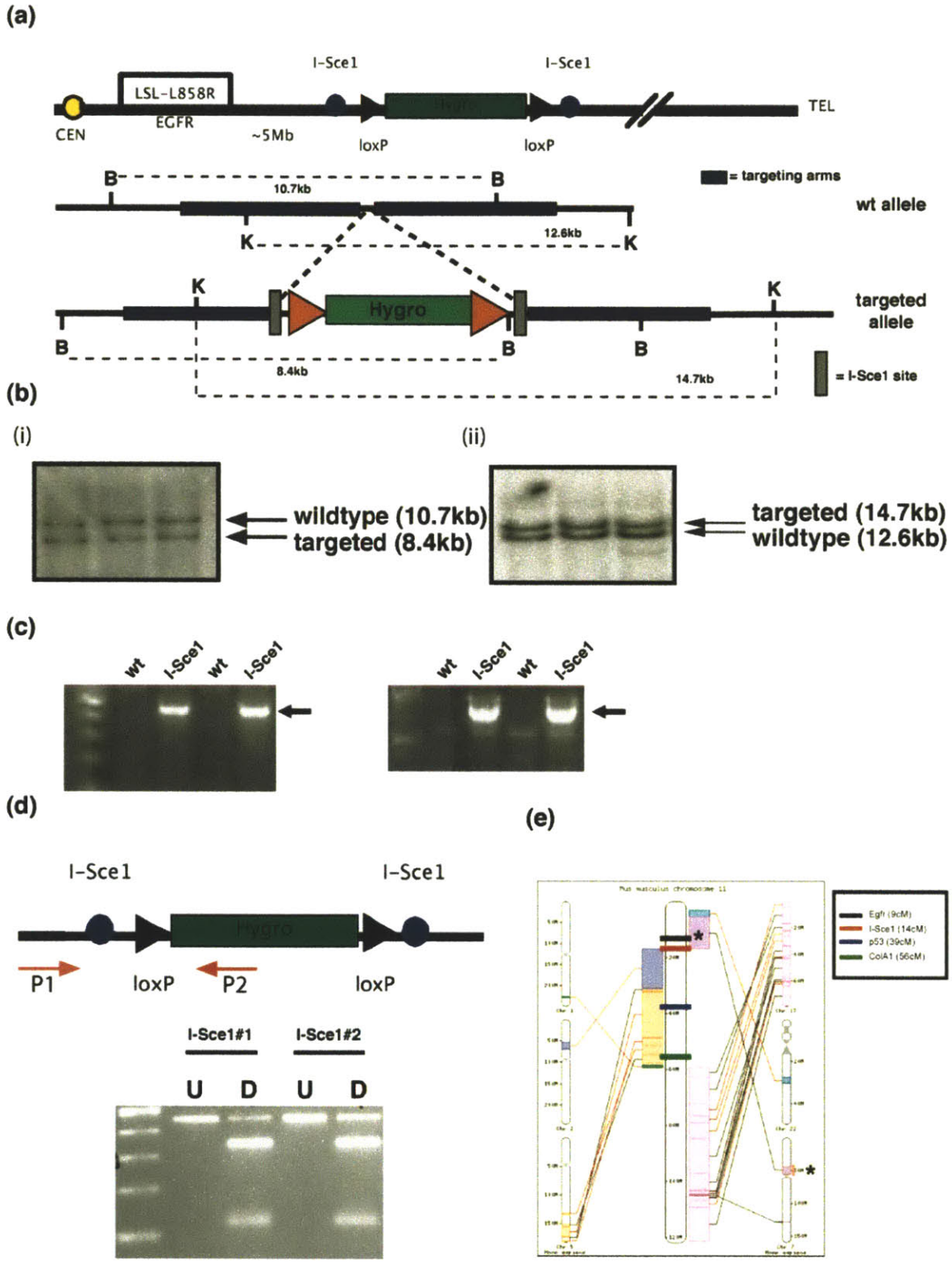
Figure 1. Proposed model for how I-Sce1 induced double strand breaks (DSBs) could generate an amplified mutant *Egfr* allele.

Cre-mediated deletion of the LSL and the hygromycin cassette would leave chromosome 11 as depicted in **(a)**. Upon entry into S-phase, chromosomes would duplicate **(b)**, and if I-Sce1 is expressed, a DSB would occur potentially leading to sister-chromatid fusion **(c)**. Due to the presence of a dicentric chromosome an anaphase bridge would result, and the ensuing tension from the centromeres being pulled towards opposite poles would cause a break to randomly occur **(d)**. If this break occurs telomeric to *Egfr*, as depicted, the gene would now be duplicated, and the broken chromosome could now enter another breakage-fusion-bridge cycle, leading to inverted repeats of mutant *Egfr*, and the capping of the break with telomeres would be one way to end the cycle **(e)**. loxP ▶, centromere (CEN) ●, telomere (TEL) ●, I-Sce1 restriction site ●.

Results

Generation and initial characterization of the I-Sce1-LHL-I-Sce1 allele

In order to create a model that would allow inducible amplification of endogenous murine genes in a targeted manner we set out to combine our *Egfr*$^{LSL\text{-}L858R}$ model (Chapter 2) with a system involving the yeast endonuclease, I-Sce1. We selected I-Sce1 as our means of introducing DSBs since its recognition sequence is absent from the mouse genome, and at the initiation of this project this enzyme had been used extensively in mammalian cell culture to investigate mechanisms of homologous recombination (Liang et al., 1998; Rouet et al., 1994; Weinstock et al., 2006). We generated a simple construct that contained a floxed hygromycin cassette, for selection in ES cells, flanked by individual I-Sce1 sites, designated as I-Sce1-LHL-I-Sce1. We targeted this construct in ES cells to a region of mouse chromosome 11 that was approximately 5Mb telomeric to the *Egfr* locus (Figure 2A). The particular position of the targeting locus was a somewhat arbitrary choice mediated by the region meeting both of the following criteria: 1) an absence of nearby genes whose expression might have been disrupted by the targeting construct or the continued presence of the hygromycin cassette which remains in place until Cre is delivered, and 2) the targeted locus needed to be a sufficient distance from the *Egfr* locus such that it would be feasible to recombine the I-Sce1 allele in *cis* with the *L858R* allele through a breeding strategy, while close enough that the probability of later separation during meiosis would be low (see Figure 2E for the final organization of chromosome 11). Southern blotting analysis of ES cells identified correctly targeted clones at both the 5' and 3' ends (Figure 2B), and germline transmission was confirmed by PCR analysis (Figure 2C). We amplified a region encompassing the I-Sce1 sites using tail DNA from these mice and subjected this to digestion with recombinant I-Sce1 *in vitro*, confirming that the I-Sce1 sites were intact in the mice (Figure 2D). We now had a tool that would allow targeted breaks telomeric to the *Egfr*


(a)
I-SceI
I-SceI
LSL-L858R
EGFR
TEL
CEN
~5Mb
loxP
loxP
= targeting arms
B
B
10.7kb
wt allele
K
K
12.6kb
K
K
Hygro
targeted allele
B
B
B
8.4kb
14.7kb
= I-SceI site
(b)
(i)
(ii)
wildtype (10.7kb)
targeted (8.4kb)
targeted (14.7kb)
wildtype (12.6kb)
(c)
wt
I-SceI
wt
I-SceI
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I-SceI
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I-SceI
(d)
(e)
I-SceI
I-SceI
Hygro
P1
loxP
P2
loxP
I-SceI#1
I-SceI#2
U
D
U
D
Egfr (9cM)
I-SceI (14cM)
p53 (39cM)
ColA1 (56cM)

Figure 2. Generation of the *I-Sce1-LHL-I-Sce1* allele.

(a) Schematic of the targeting strategy for the *I-Sce1-LHL-I-Sce1* allele. Note: The *Egfr* locus is approximately 5Mb centromeric to the targeted region as shown. B=BamH1 site, K=Kpn1 site. **(b)** Southern blotting analysis of positive ES clones using probes for the (i) 5' end on DNA digested with BamH1, and the (ii) 3' end on DNA digested with Kpn1. **(c)** Confirmation of germline transmission using PCR primers designed against the (i) 5' end and the (ii) 3' end of the allele. Product from the *I-Sce1-LHL-I-Sce1* allele is indicated by an arrow. Note: this PCR does not detect the wild-type allele. **(d)** Tail DNA from mice identified in (c) as having germline transmission of the allele was amplified with the PCR primers P1 and P2 as shown. After gel purification this PCR product was digested with I-Sce1 *in vitro* to confirm the presence of the I-Sce1 site. **(e)** Schematic of mouse chromosome 11 from the NCBI comparative genomics website, showing the relative positions of the genes used in this study that are found on chromosome 11. A comparative analysis highlights that the region surrounding human *EGFR* on chromosome 7 is syntenic with the region surrounding mouse *Egfr* on chromosome 11, and is designated by a *.

locus, and importantly, as shown in Figure 2E the region of human chromosome 7 that is typically amplified in human tumors is syntenic with the region of mouse chromosome 11 that lies between *Egfr* and the I-Sce1 cassette.

Optimization of strategies for I-Sce1 expression in vivo

Having established germline transmission of the *I-Sce1-LHL-I-Sce1* allele we proceeded to generate a colony that was $Egfr^{-LSL\text{-}L858R/+}$- *I-Sce1-LHL-I-Sce1*-$p53^{FL/FL}$ (from now on referred to as *EIP*, and unless otherwise stated the *I-Sce1-LHL-I-Sce1* allele is always in *cis* to the mutant *Egfr* allele). Prior to initiating experiments *in vivo* we sought to assess whether we could induce DSBs in cell culture, and for this purpose generated MEFs that were either *EIP* or $p53^{FL/FL}$. Published reports employing I-Sce1 had typically used transient transfection to achieve expression of the enzyme. We wanted to maintain expression and possibly employ inducible systems and so we began by constructing basic retroviral and lentiviral expression vectors to express I-Sce1. We could not achieve expression levels of I-Sce1 that reflected those achieved using transient transfection (data not shown), either in *EIP* or $p53^{FL/FL}$ MEFs, confirming that this was not simply due to selection against high level I-Sce1 expression in cells with a target restriction site. Upon further examination we realized that the I-Sce1 cDNA had been optimized for expression in *E.Coli* and yeast. We chose to get the cDNA codon-optimized specifically for use in murine cells and went on to compare the expression level of the optimized version (I-Sce1°) with the original (I-Sce1) from the same vector. As shown in Figure 3A the codon-optimized version was expressed to significantly higher levels upon retroviral or lentiviral infection of MEFs. This bifunctional lentiviral vector was modified from the original that has been successfully used to express luciferase *in vivo* (DuPage et al., 2011) and is composed of the Ubc promoter driving I-Sce1° and the Pgk promoter driving Cre expression.

We hypothesized that an inducible system might be preferable so that all of the

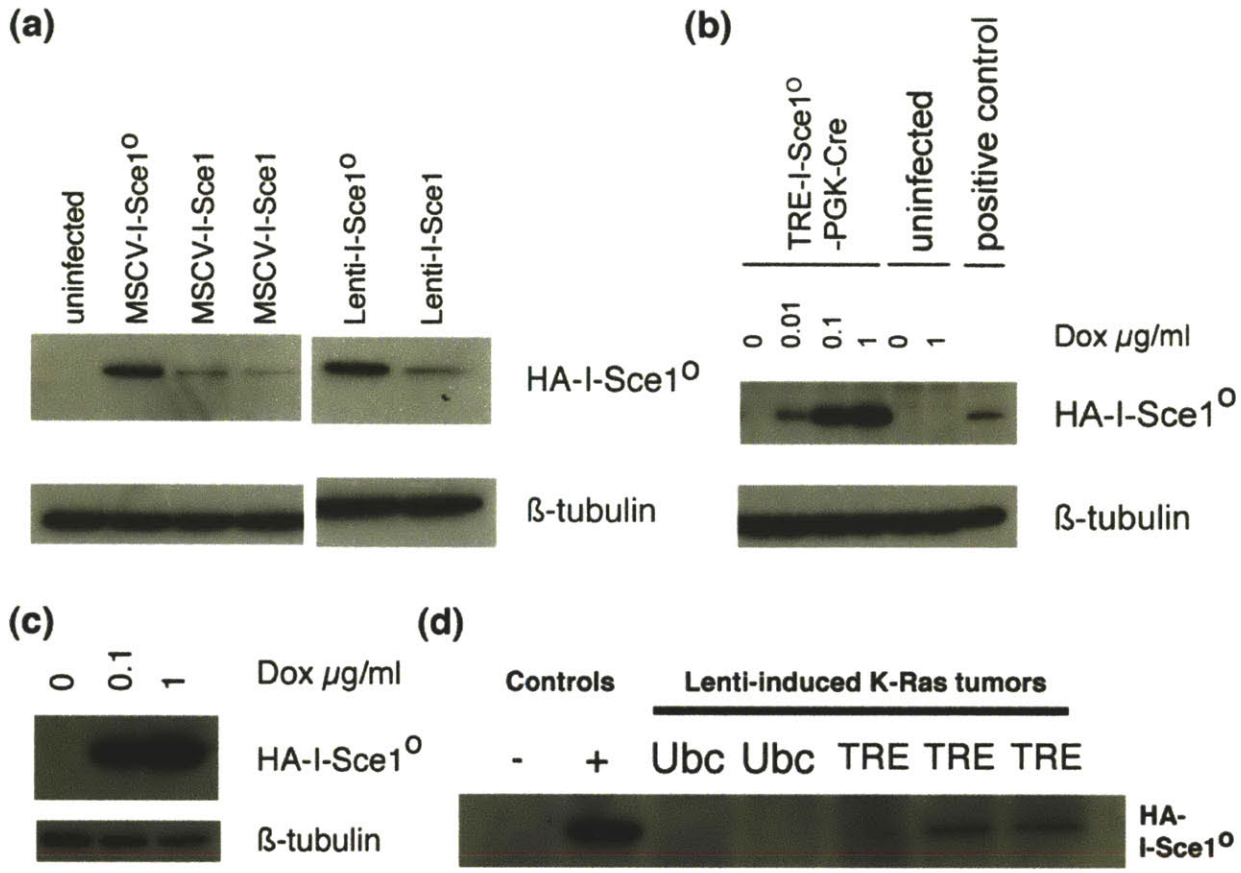


Figure 3. Codon optimization of I-Sce1 and lentiviral expression strategies.

Western blot analysis of approaches to express I-Sce1. **(a)** Comparison of I-Sce1 and codon-optimized I-Sce1^{o} expression upon retroviral or lentiviral infection of MEFs. Due to the presence of a tag, I-Sce1 is detected using an antibody against HA. **(b)** Inducible expression from tre-I-Sce1^{o}-Pgk-Cre lentivirus upon infection of HeLa Tet-On cells. Positive control is from (a). **(c)** Inducible expression from tre-I-Sce1^{o}-UbcrtTA lentivirus in wild-type MEFs. **(d)** I-Sce1^{o} expression in tumors from *LSL-K-Ras^{G12D}; p53$^{FL/FL}$* mice infected with Ubc-I-Sce1^{o}-PgkCre (Ubc) or in tumors from *LSL-K-Ras^{G12D}; p53$^{FL/FL}$; CCSP-rtTA* mice infected with tre-I-Sce1^{o}-Pgk-Cre (TRE). Controls lysates are from (b).

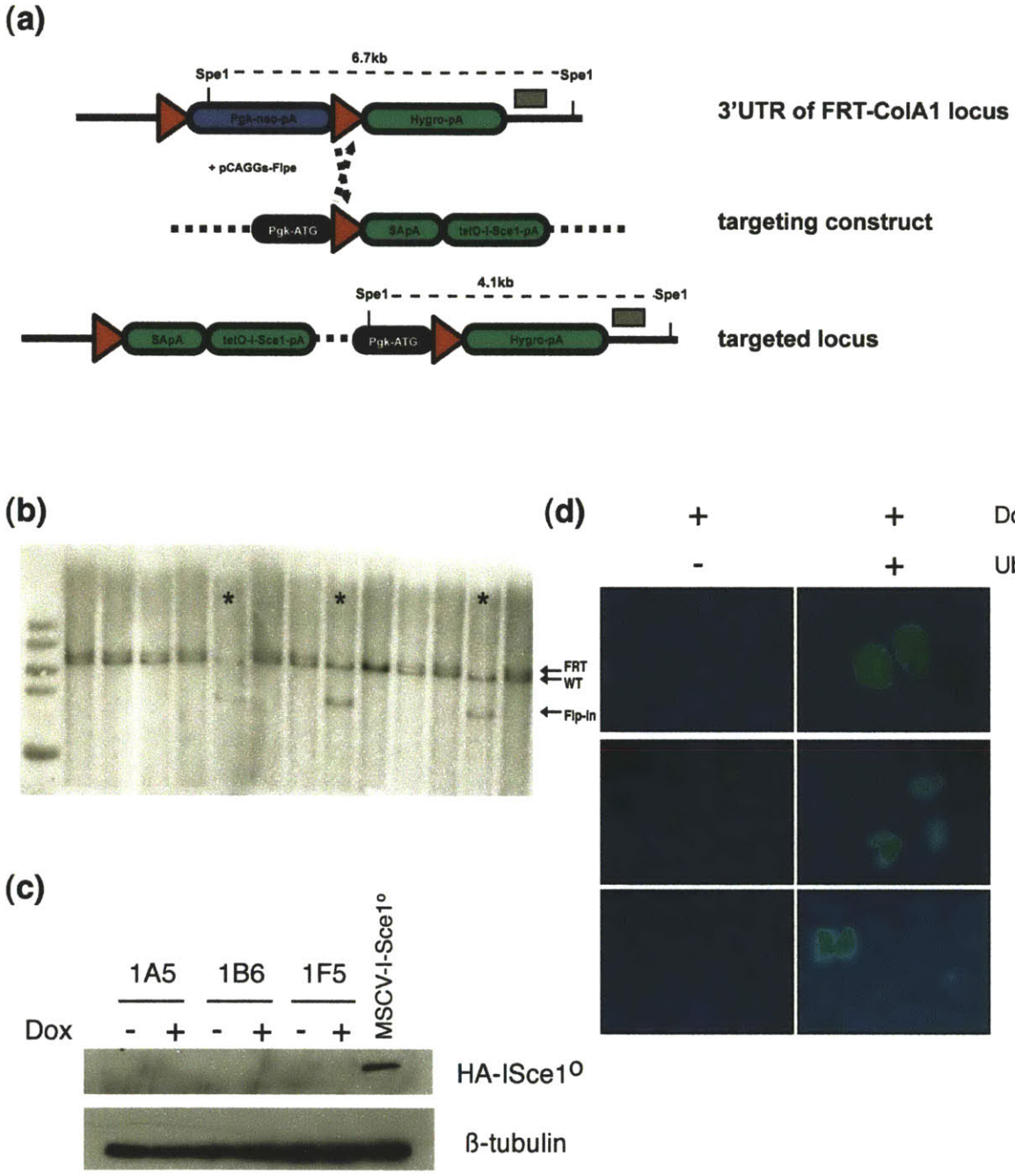
necessary Cre-mediated recombination events could take place prior to the induction of I-Sce1 breaks. A lentiviral vector was constructed that combined tre-I-Sce1^{o} with Pgk-Cre, and we showed that following infection of HeLa cells which stably express rtTA, I-Sce1^{o} expression could be induced with the addition of doxycycline (Figure 3B). Finally we wished to create a vector that would allow us to inducibly express I-Sce1^{o} in MEFs and we generated another lentiviral construct composed of tre-I-Sce1^{o}-Ubc-rtTA-ires-blasticidin, and once again upon introduction into MEFs I-Sce1^{o} was robustly induced upon doxycycline addition (Figure 3C).

Having established lentiviral expression vectors that worked *in vitro*, we now wanted to determine whether they could be used to express I-Sce1^{o} in the lung, and to a level sufficient to induce DNA breaks. The low number of cells infected by intratracheal delivery of lentiviruses precluded the use of western blotting analysis of whole lung to determine I-Sce1^{o} expression levels post infection, and we also wanted to test the expression in a system that does not contain the *I-Sce1-LHL-I-Sce1* cassette in case there was selection against cutting and hence expression. We took advantage of the *LSL-K-Ras^{G12D}, p53$^{FL/FL}$ (KP)* model where tumors of sufficient size for protein analysis are induced within a 16-week timeframe upon Cre addition (Jackson et al., 2001). We infected *KP* mice with the constitutive Ubc-I-Sce1^{o} lentivirus, and 16-24 weeks post-infection tumors were collected, but our western blot analysis failed to detect any enzyme in these tumors (Figure 3D). In parallel we tested the inducible lentiviral vectors in *KP* mice that also had a lung specific *CCSP-rtTA* allele, and we administered doxycycline in the diet two weeks prior to harvesting the tumors. We found low but variable protein expression using the doxycycline-inducible lentivirus (Figure 3D), but the I-Sce1 mRNA was detectable in these tumors (Figure 4E). This inducible lentiviral vector has subsequently been used to successfully express other cDNAs in the lung, although similar issues with variable expression have emerged (Meylan et al., 2009). Similar issues of western blot detection associated with the expression of other cDNAs in the lung using the same constitutive or inducible lentiviral vectors have been encountered, but in both cases the proteins were readily detected by other means

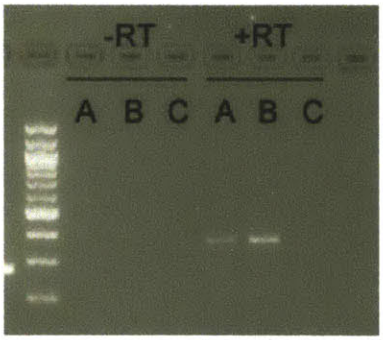
(DuPage et al., 2011; Meylan et al., 2009). We concluded that these expression difficulties were not a specific issue with I-Sce1°, but since we had no alternative assay for I-Sce1° expression we had to assume I-Sce1° was expressed and proceed with these vectors. We also had no scale against which to assess I-Sce1° expression to determine what would be an optimum amount. We infected a cohort of *EIP* mice with the Ubc-I-Sce1°-Pgk-Cre lentivirus and aged them for 18-24 months. We also infected a cohort of *EIP; CCSP-rtTA* mice with the doxycycline inducible lentivirus, and administered doxycycline food one week after lentiviral infection for a period of 4-6 months. Neither group developed tumors.

Generation of an inducible I-Sce1° knock-in mouse

Our disappointing results with the lentiviral approach caused us to re-evaluate our strategy. We reasoned that we needed to achieve improved expression of I-Sce1°, and due to certain technical and biological issues in the lung we sought to extend our studies to include other tissues. To this end we generated a doxycycline-inducible I-Sce1° construct that was targeted to the *ColA1* locus using a FLP-in strategy (Figure 4A). Expression from this locus has been previously characterized *in vivo* using a *tetO-GFP* combined with a *R26-M2rtTA* allele, and apart from the skeletal muscle and the brain GFP-positive cells were detected in all tissues, albeit at different levels depending on the cell type (Beard et al., 2006). We targeted ES cells, and positive clones were selected by southern blotting and injected (Figure 4B). Due to the difficulties we had with I-Sce1° expression using lentiviral vectors we wanted to confirm that this system would resolve these issues. We took three positive ES cell clones, transfected an rtTA expressing plasmid and assessed I-Sce1° expression in the presence of doxycycline (Figure 4C). Once again we failed to detect I-Sce1°, although poor transfection efficiency of ES cells may have contributed to this. We repeated this experiment but this time monitored I-Sce1° expression using immunofluorescence (Figure 4D). In the presence of rtTA and doxycycline we finally detected I-Sce1° expression, which was properly localized to the nucleus in all clones.


(a)
6.7kb
Spe1
Spe1
Pgk-neo-pA
Hygro-pA
3'UTR of FRT-ColA1 locus
+ pCAGGs-Flpe
Pgk-ATG
SApA
tetO-I-Sce1-pA
targeting construct
4.1kb
Spe1
Spe1
SApA
tetO-I-Sce1-pA
Pgk-ATG
Hygro-pA
targeted locus
(b)
*
*
*
FRT
WT
Flp-in
(c)
1A5
1B6
1F5
MSCV-I-Sce1°
Dox
- + - + - +
HA-ISce1°
ß-tubulin
(d)
+ + Dox
- + Ubc-rtTA
1A5
1B6
1F5

(e)

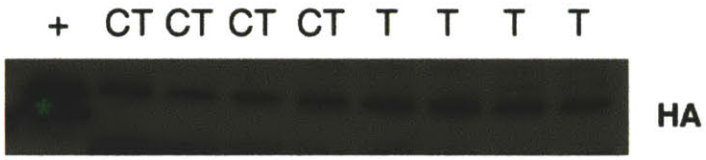


A = CCSPrtTA; tetO-I-Sce1^{O} lentivirus + Dox

B = CCSPrtTA; ColA1-tetO-I-Sce1^{O} + Dox

C = ColA1-tetO-I-Sce1^{O} + Dox

(f)



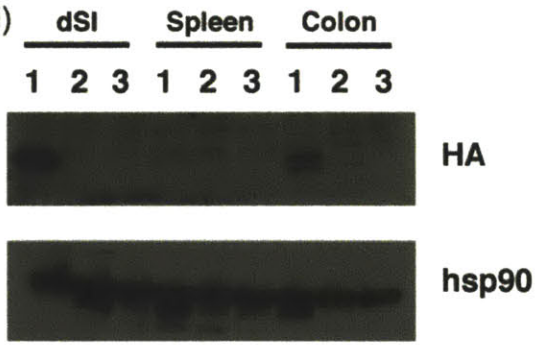
C = CCSPrtTA

T = ColA1-tetO-I-Sce1^{O}

(g)

(i)

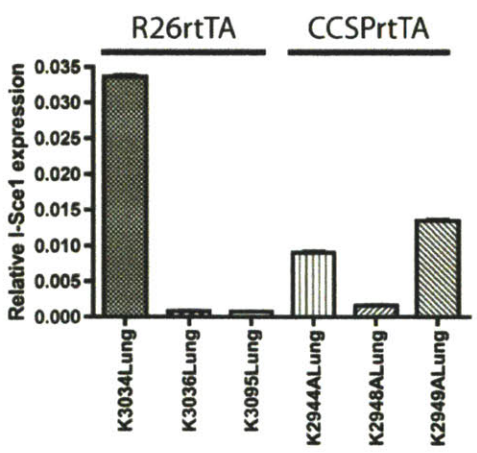
(ii)



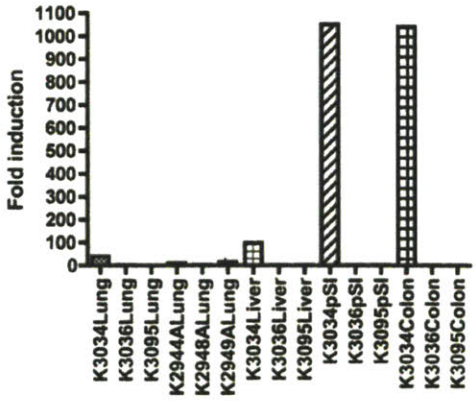
1 = EIP; R26rtTA; ColA1-tetO-I-Sce1^{O}; + Dox
2 = +/+; + Dox
3 = R26rtTA; ColA1-tetO-I-Sce1^{O}; no Dox

(h)

(i)



(ii)



K3034 = EIP; R26rtTA; ColA1-tet-I-Sce1; Dox 13d

K3036 = EIP; R26rtTA; Dox 13d

K3095 = wt; Dox 13d

K2944 = EIP; CCSPrtTA; ColA1-tet-I-Sce1; Dox 20d

K2948 = EIP; CCSPrtTA; ColA1-tet-I-Sce1; no Dox

K2949 = EIP; CCSPrtTA; ColA1-tet-I-Sce1; Dox 20d

Figure 4. Generation and characterization of a *ColA1-tetO-I-Sce1^{o}* allele.

(a) Targeting strategy for *ColA1-tetO-I-Sce1^{o}* allele. Position of southern probe is designated by ■ and Spe1 sites used are indicated. **(b)** Southern blot analysis of ES clones. Spe1 digested DNA was probed with 3'probe ■ and the band sizes were wt: 6.2kb, FRT-Pgk-neo: 6.7kb, Flp-in: 4.1kb. Three positive clones containing the *Flp-in* allele are indicated by *. **(c)** Failure to detect tetO-I-Sce1^{o} induction in ES cells transfected with a UbcrtTA expressing plasmid. MEFs infected with MSCV-I-Sce1^{o} serve as the positive control. **(d)** Immunofluorescence detection of I-Sce1^{o} expression in ES clones from (c). **(e)** Detection of I-Sce1^{o} expression by qPCR in lung tumors infected with a tet-inducible lentivirus or in lungs from *ColA1-tetO-I-Sce1^{o}* mice. **(f)** Absence of protein expression in *CT* or *T* lungs from mice treated with doxycycline. Positive control is indicated with a *. **(g)** (i) Western blot on lung tissue for I-Sce1^{o} over a timecourse of doxycycline administration in *CCSPrtTA; ColA1-tetO-I-Sce1^{o}* mice. Positive control is indicated with a *. (ii) Western blot detection of expression from *ColA1-tetO-I-Sce1^{o}* in the intestine of 6-8 week old mice administered doxycycline for 2-3weeks as indicated. **(h)** qPCR analysis of *ColA1-tetO-I-Sce1^{o}* expression assessed by SYBR Green. (i) a comparison of expression in the lung using *R26rtTA* or *CCSPrtTA*, and (ii) a comparison across tissue types confirming high-level expression in the intestine. The same mice were used in parts (g)(ii) and (h).

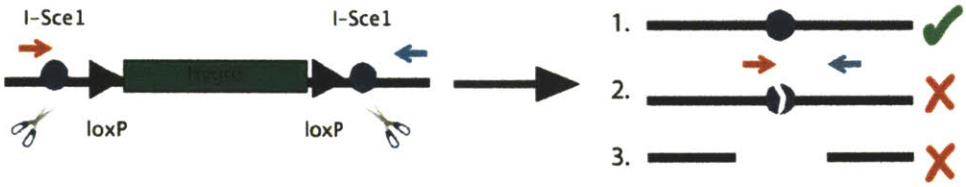
ColA1-tetO-I-Sce1° *(T)* transgenic animals were generated and combined with *CCSP-rtTA (C)* to test induction. We found we could detect I-Sce1° expression at the mRNA level in the lungs of *CT* mice after one week of a doxycycline diet, and the allele did not appear to be leaky (Figure 4E). However, once again we failed to detect I-Sce1° using western blotting in a panel of lungs from *CT* or *T* mice treated with doxycycline for one week (Figure 4F). We hypothesized that I-Sce1° might be inducing spurious breaks leading to silencing of expression and so we tested whether we could detect I-Sce1° in the lung at earlier time points post doxycycline addition, but met with no success (Figure 4G, i). Given that one of our initial motivations for creating this allele was to target I-Sce1° expression elsewhere, we combined the transgene with the *R26rtTA (R)*, assessed induction in a series of tissues, and found robust induction in the intestine and marginal induction in the spleen (Figure 4G, ii). Having established that the allele could indeed be induced, we carried out qPCR for I-Sce1° to determine whether relative mRNA levels could explain the difference at the protein level. Both *CT* and *RT* mice produced detectable increases in I-Sce1° mRNA above background in the lung (Figure 4H, i), but when the level of expression in K3095Lung (wild-type) was set to a baseline value of one and all tissues were compared on a relative scale, it was grossly apparent that the induction was vastly superior in the proximal small intestine and the colon compared to the lung and liver, in line with our data at the protein level (Figure 4H, ii).

Assessment of the induction of DNA breaks in the context of an in vivo setting

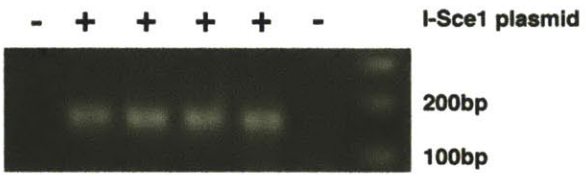
We next set out to determine whether the levels of I-Sce1 expression were sufficient to induce cutting at the I-Sce1 sites. The design of the *I-Sce1-LHL-I-Sce1* allele did not incorporate any feature that would allow us to tell if a break had occurred and been accurately repaired, and so we reasoned the only measurement we could make was of I-Sce1 having induced breaks at both sites in a temporally narrow time window such that rather than being resealed individually, the intervening hygromycin cassette was lost and a single I-Sce1 site was restored. We

(a)

(i)

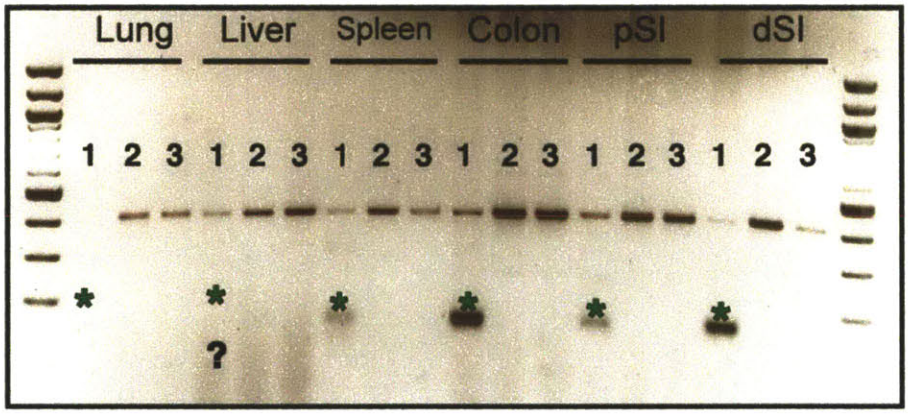


(ii)



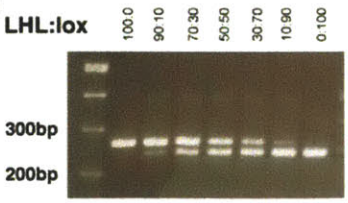
(b)

(i)



1 = EIP; R26rtTA; ColA1-tetO-I-Sce1; Dox 19d
2 = +/+; Dox 19d
3 = EIP; R26rtTA; ColA1-tetO-I-Sce1; no Dox

(ii)



(iii)

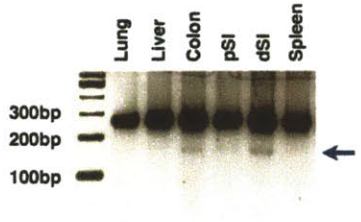


Figure 5. Detection of I-Sce1 induced DSBs in cells and in tissues.

(a) (i) Schematic of the assay used to detect induction of DSBs. Primers used are indicated by an arrow, and they will only produce a product in case 1, where both sites are cut and then resealed, and not in cases 2, or 3 where the site is cut or has been processed to eliminate the I-Sce1 site, or in the presence of the LHL cassette. (ii) PCR on *I-Sce1-LHL-I-Sce1/+* MEFs transfected with an I-Sce1 expression plasmid reveals an approximately 161bp band corresponding to loss of the LHL. **(b)** (i) DSB assay was carried out on tissues from mice of the indicated genotypes. A band of the correct size is indicated by * and is present but faint in the lung and perhaps liver lanes. The upper band is a contaminating band that appears under all conditions. (ii) Semi-quantitative PCR assay to distinguish the LHL from the lox cassette. (iii) The PCR reaction from part (ii) was carried out on DNA from tissues from mouse #1 but the predominant band was the LHL and only a faint band corresponding to the single I-Sce1 site, indicated by the blue arrow, could be detected in some lanes.

designed primers flanking the cassette, with one primer partially overlapping the 5' I-Sce1 site to prevent any amplification of the wild-type allele (Figure 5A, i). As depicted in Figure 5A (i) this assay will only detect the double break scenario, and only if the site is restored or at minimum sufficiently restored to allow primer binding. We began by transfecting I-Sce1 into MEFs that contained only the *I-Sce1-LHL-I-Sce1* cassette, and 96hrs later harvested DNA and carried out the PCR reaction as indicated. Due to the selected PCR conditions and size of the LHL cassette there was no product produced with the intact allele. A product was only detected in cells that had been exposed to I-Sce1, and the results of sequencing a sample of these PCR products found that 9 of 16 had a perfectly restored I-Sce1 site (Figure 5A, ii).

Having confirmed cutting *in vitro* we wanted to assess the cutting frequency *in vivo,* and to assess whether the cutting frequency between different tissues was a reflection of the expression level of *ColA1-tetO-I-Sce1*^{o}. We harvested tissues from *EIP; R26rtTA; ColA1-tet-I-Sce1*^{o} mice with and without 19 days of doxycycline administration, and we repeated the PCR reaction, as in MEFs, on DNA harvested from whole lung, liver, spleen, colon, proximal small intestine, and distal small intestine. We detected cutting in all tissues and it correlated with our previous data on relative I-Sce1^{o} expression levels (Figure 5B, i). We sought to assess the structure of the locus after this cutting had occurred to determine whether the I-Sce1 site was still intact or had been repaired such that no more cutting could occur. A caveat of this analysis is that we couldn't monitor the dynamics of this cutting process over the course of the three-week period of the experiment and as always we could never detect the breaks that occur but are repaired correctly. We purified the PCR products from each tissue, including liver and lung where the bands were almost undetectable. The sequencing results verified the loss of the hygromycin cassette and revealed to us that the remaining sites were distributed in an approximately 2:1 ratio between accurately repaired and misrepaired sites that were no longer a substrate for I-Sce1 (data not shown). We wanted to investigate the frequency of breaks using a three-primer PCR reaction, and found that we could distinguish between different ratios of the

intact LHL and the recombined lox cassette (Figure 5B, ii), but having carried out this same reaction on tissues the single I-Sce1 site band represented a minor fraction of the total if it was detectable at all (Figure 5B, iii). While acknowledging the severe limitations of the assay, our interpretation of these results was that while breaks can be induced by I-Sce1^{o} at the targeted region, that they are not a frequent event, specifically in the target tissue, the lung, and because of this we reasoned that we had to maximize the other experimental conditions to try to compensate for this unexpected low cutting frequency.

Circumventing repair mechanisms and low proliferation rates in vivo

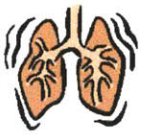
We chose to try and model amplification in different tissues as a consequence of the issues we encountered with the expression and function of I-Sce1^{o} in the lung. No one tissue satisfied all of the original criteria we set out to attain, with each having distinct advantages and disadvantages. In each case we used cohorts of *EIP; Xrcc4$^{FL/FL}$; ColA1-tetO-I-Sce1^{o}* and *p53$^{FL/FL}$; Xrcc4$^{FL/FL}$; ColA1-tetO-I-Sce1^{o}* mice. We chose to incorporate the *Xrcc4* floxed allele into our system as a means to impair repair mechanisms. Xrcc4 is a component of the NHEJ complex involved in non-HR mediated repair of DSBs, and *Xrcc4* null mice are viable only in combination with *p53* loss, with compound mutant mice succumbing to B-cell lymphoma containing amplified *c-Myc*, or medulloblastoma containing amplification of *N-Myc* or *CyclinD2* when combined with a Nestin-Cre transgene (Gao et al., 2000; Yan et al., 2006).

Due to the obvious relevance to NSCLC our primary goal was to achieve *Egfr* gene amplification in the lung. We took a multitude of approaches to try to overcome some of the technical limitations associated with carrying out these experiments in the lung. We began by combining the genotypes shown in Figure 6A in conjunction with *CCSP-rtTA* with either tet-inducible I-Sce1^{o} lentiviruses or the *ColA1-tetO-I-Sce1^{o}* allele, and Cre was delivered using either a lenti- or adenovirus. Apart from the question of relevance, the other major reason as to why we pursued amplification strategies in the lung was on the basis of the known tumor

(a)

$Egfr^{LSL\text{-}L858R/+}$-I-Sce1^{LHL}-I-Sce1-$p53^{FL/FL}$; $Xrcc4^{FL/FL}$
or
$p53^{FL/FL}$; $Xrcc4^{FL/FL}$

(i)

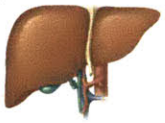


Cre delivery: Adenovirus, Lentivirus
I-Sce1 expression: Viral or tetO-I-Sce1°
Dox induction: CCSPrtTA

Advantages: ✔
- Relevance to NSCLC with EGFR amplification
- Known phenotype upon mutant EGFR overexpression
- Can induce proliferation using naphthalene

Disadvantages: ✘
- Difficulty expressing I-Sce1°
- Low basal proliferation rate

(ii)

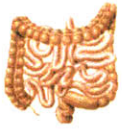


Cre delivery: Adenovirus tail-vein
I-Sce1 expression: tetO-I-Sce1°
Dox induction: R26rtTA

Advantages: ✔
- Cre accesible
- L858R mice develop hepatomas
- Induce proliferation with CCl_4

Disadvantages: ✘
- Low proliferation rate
- tetO-I-Sce1° poorly expressed
- No relevance to human HCC

(iii)

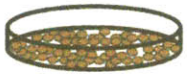


Cre delivery: Villin-Cre
I-Sce1 expression: tetO-I-Sce1°
Dox induction: R26rtTA

Advantages: ✔
- High basal proliferation rate
- No injury needed
- tetO-I-Sce1° highly expressed

Disadvantages: ✘
- No relevance
- Unknown if Egfr overexpression would result in a phenotype
- VillinCre is not restricted to the intestine
- Viral delivery not feasible

(b)



$Egfr^{LSL\text{-}L858R}$-I-$Sce1^{LHL}$-I-Sce1-$p53^{FL/FL}$
R26-CreER/R26-CreER
MEFs

Cre delivery: CreER, inducible
I-Sce1 expression: Viral - constitutive or dox inducible
Dox induction: UbcrtTA on lentiviral vector

Advantages: ✔
- **Cells are highly proliferative**
- **Breaks can be induced in much larger numbers of cells than possible *in vivo***
- **High levels of I-Sce1 expression can be achieved**
- **Potential co-operating events can be induced with shRNAs**

Disadvantages: ✗
- **System is not relevant to NSCLC**
- **Difficult/impossible to select out cells with amplified *Egfr***

Figure 5. Strategies to engineer gene amplification (a) in the mouse or (b) in cells in culture.

Advantages and disadvantages corresponding to each tissue/approach are given.

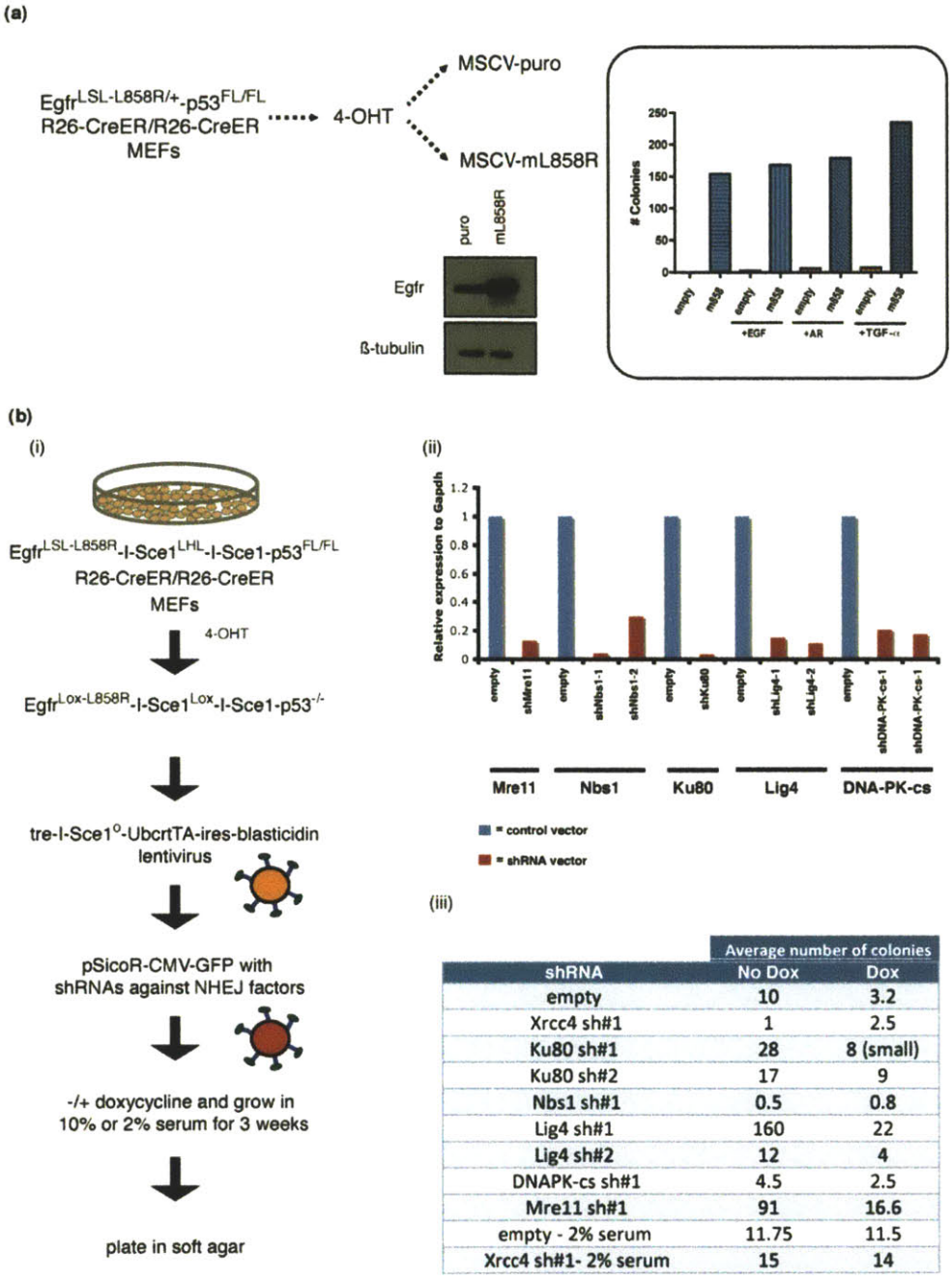
phenotype elicited by overexpression of mutant Egfr in transgenic models (Ji et al., 2006; Politi et al., 2006) or by using lentiviral delivery strategies (Chapter 2). In addition to the difficulty of achieving high I-Sce1^{o} expression levels, the other outstanding issue with the lung is the low basal proliferation rate. Since cells have to transition through S-phase with a break to initiate BFB cycles we sought to augment the proliferation rate in the lung by treating mice with naphthalene, a chemical known to specifically ablate Clara cells in the lung and stimulate renewal of the lung epithelium (Reynolds et al., 2000). Naphthalene treatment was typically carried out one week post Cre-mediated recombination of the required alleles and doxycycline administration was initiated 48-72hrs later. So far we have been unsuccessful in engineering lung tumors with any combination of these approaches.

As a result of the germline mutant *Egfr* mice succumbing to low-grade hepatomas, albeit with a long latency, we sought to take advantage of this mutant Egfr-induced phenotype in the context of our amplification strategy. As depicted in Figure 6A, (ii), we targeted the liver using *R26rtTA* in combination with *ColA1-tetO-I-Sce1^{o}*, and induced recombination via tail-vein injection of adenovirus-Cre. Mirroring the situation with the low mitotic index in the lung our options to increase this in the liver were either to perform partial hepatectomies, or to administer carbon tetrachloride, CCl_4, an agent known to induce significant hepatocyte injury and subsequent proliferation and repair in the liver (Gupta et al., 1999; Moh et al., 2007). We chose the latter primarily due to the ease of administration and low mortality rate associated with it. We acknowledge that while the specific relevance of a potential model of mutant *Egfr* amplification to human hepatocellular carcinoma is minimal, EGFR signaling is known to be important in liver physiology and aberrant expression of EGFR has been documented in HCC (Keng et al., 2009; Natarajan et al., 2007). This strategy has so far failed to accelerate hepatoma development in these mice, although due to the long latency these experiments are still in progress. For our final *in vivo* approach we selected the intestine for two simple reasons, (i) the high basal proliferation rate which obviated the need for any injury-mediated proliferation, and (ii) the

relatively high expression level of the *ColA1-tetO-I-Sce1°* transgene, with the associated increased frequency of induced breaks (Figure 6A, iii). We combined our standard genotype with *R26rtTA* and Cre expression was mediated by another transgene, *Villin-Cre*, as the intestine is not readily accessible for viral delivery. These experiments were initiated but due to the non-intestinal specific nature of the *Villin-Cre* allele, both the control and experimental animals succumbed to a kidney-related defect within a few months, halting the experiment.

A cell culture based strategy to identify cells with Egfr amplification

Although our initial approach had focused only on the induction of amplification in an *in vivo* setting, we realigned our goals due to the many technical hurdles that we were faced with, and chose to try and elicit amplification in cell culture. The advantages of this approach were as outlined in Figure 6B, and included the ease with which I-Sce1° could be overexpressed, the large number of cells that could be screened, and the fact that the cells were constantly proliferating. We made use of MEFs we had isolated from *EIP; R26*$^{CreER/CreER}$ mice; *R26-CreER* is a tamoxifen-regulatable version of Cre expressed from the ubiquitously expressed *Rosa26* promoter such that it is sequestered in the cytoplasm by heat shock proteins until the addition of 4-OHT, upon which it traffics into the nucleus and induces recombination (Ventura et al., 2007). Unlike the *in vivo* setting we realized that we needed an assay that would allow us to screen large numbers of MEFs and identify those with higher than normal levels of Egfr. To model *Egfr* amplification we prepared *EP* MEFs infected with either MSCV-puro-empty or MSCV-puro-Egfr^{L858R} (Figure7A) and tested a battery of methods including sorting cells using Alexa-Fluor 647-labeled EGF or growing cells under different serum conditions to identify the conditions that gave the most robust difference between the cell types. We settled on growth in soft agar as our means to screen for clones that overexpressed the receptor as 1) in the absence of additional ligand, the average number of colonies obtained in the control was <1 per 50,000 cells plated (Figure 7A), 2) this method was amenable to screening large numbers of cells, and 3) growth in



	Average number of colonies	
shRNA	No Dox	Dox
empty	10	3.2
Xrcc4 sh#1	1	2.5
Ku80 sh#1	28	8 (small)
Ku80 sh#2	17	9
Nbs1 sh#1	0.5	0.8
Lig4 sh#1	160	22
Lig4 sh#2	12	4
DNAPK-cs sh#1	4.5	2.5
Mre11 sh#1	91	16.6
empty - 2% serum	11.75	11.5
Xrcc4 sh#1- 2% serum	15	14

Figure 6. Cell culture strategy to identify amplification.

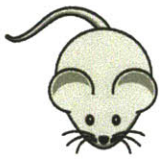
(a) Test strategy using MEFs overexpressing Egfr^{L858R} as indicated by western blotting. Number of colonies that form in soft agar between single copy L858R and cells that overexpress L858R under a range of conditions. **(b)** (i) Actual strategy to identify cells with amplification, (ii) qPCR validation of knockdown by hairpins targeting components of the NHEJ pathway, and (iii) table showing the results of the screen, with the hairpin target if applicable, and the number of colonies formed in the presence or absence of doxycycline, which corresponds to the presence or absence of I-Sce1^{o} expression.

soft agar is the one assay for transformation that correlates with tumor growth *in vivo*. We pursued the strategy outlined in Figure 7B, (i) using doxycycline-regulated lentiviruses to express I-Sce1^{o} and this was supplemented with infection with a lentiviral vector, pSicoR-U6shRNA-CMVGFP, that allowed constitutive expression of shRNAs against components of the NHEJ pathway. These hairpins were validated for knockdown at the mRNA level (Figure 7B, ii) and we chose this strategy rather than using the *Xrcc4* floxed allele as it allowed us to try hairpins targeting many different components of the repair machinery. In the end the screen was carried out in *EIP* cells, in the presence or absence of doxycycline to induce I-Sce1^{o} expression, and in combination with a series of NHEJ-targeted shRNAs. In some cases we cultured cells in media containing 2% serum as we found that the relative difference in proliferation rate between MEFs overexpressing mutant EGFR and those expressing a single copy is much greater in 2% than 10% (data not shown). The results of the screen are outlined in Figure 7B, (iii). Surprisingly, we found an almost uniform decrease in the number of colonies that formed in the presence of I-Sce1^{o} expression, and in the context of Ligase4 and Mre11 knockdown this difference was very striking. This result ran contrary to our expectations, and while we analyzed further some of the colonies that emerged we could not find any detectable increase in Egfr levels (data not shown).

An in vivo complementation strategy using oncogenic K-Ras

Our final attempt to induce targeted amplification of mutant *Egfr* focused again on the lung, and was inspired by the universal oncogene dependence of tet-regulatable mouse models of cancer. In conjunction with poor expression of I-Sce1^{o} in the lung, the low mitotic index restricted the number of potential cells that could be targets for BFB cycles. To circumvent the issue of low numbers of cells in S-phase we decided to try to do an *in vivo* complementation experiment as outlined in Figure 8A. We wanted to induce proliferation in the *EIP* strain with a

(a)

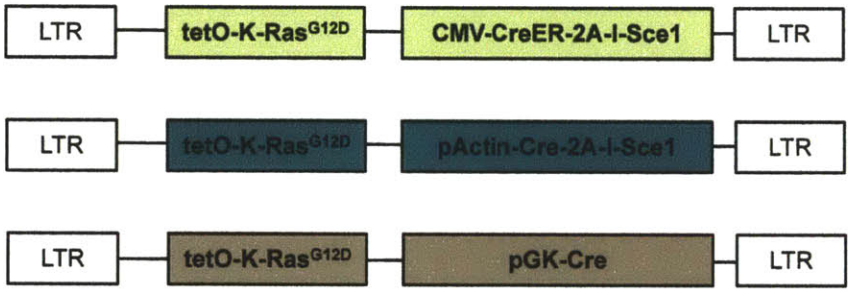


CCSPrtTA; Egfr$^{LSL-L858R/+}$-I-Sce1^{LHL}-I-Sce1-p53$^{FL/FL}$; Xrcc4$^{FL/FL}$

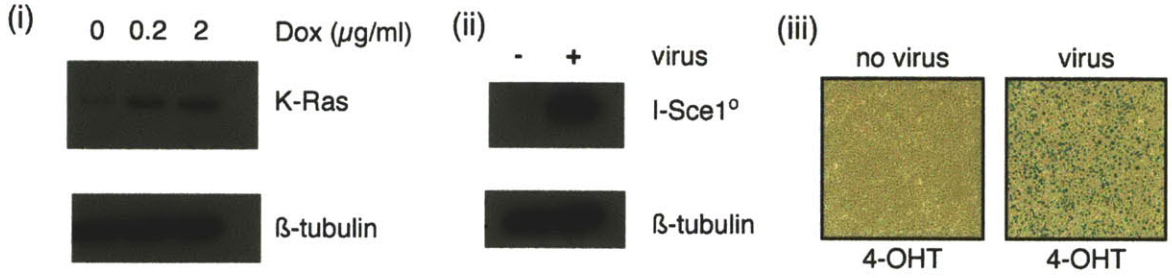
- Dox → Tumor regression due to loss of K-Ras^{G12D} expression → ??? → If Egfr amplification occurs can it complement for loss of oncogenic K-Ras?

Tumors driven by tetO-K-Ras^{G12D}
Cells in S-phase
I-Sce1^{o} expressed

(b)



(c)



(d)

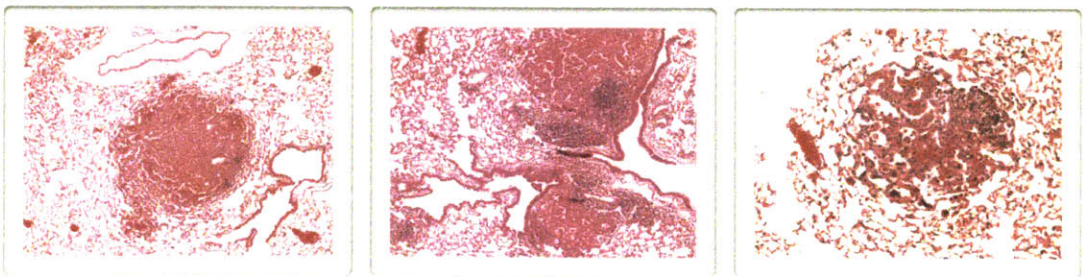


Figure 7. *In vivo* complementation strategy.

(a) Basic strategy and genotypes used. **(b)** Three lentiviral vectors that were constructed and tested for the ability to induce tumors in mice using tre-K-Ras^{G12D}. **(c)** (i) Inducible K-Ras^{G12D} expression was tested by infection of HeLa Tet-On cells, (ii) I-Sce1^{o} expression, where applicable, was confirmed by infection of MEFs, and (iii) Cre-ER expression was confirmed by infection of 3TZs, which express a conditional lacZ, in the presence or absence of 4-OHT. **(d)** Representative images of tumors in H&E stained sections of lungs from *CCSPrtTA; p53$^{FL/FL}$; ColA1-tetO-I-Sce1^{o}/ ColA1-tetO-I-Sce1^{o}* mice 7 weeks post-infection with the tre-K-Ras^{G12D}-Pgk-Cre lentivirus. **(e)** microCT images. Removal of doxycycline leads to rapid tumor regression in tumors induced with the tre-K-Ras^{G12D}-Pgk-Cre lentivirus. Areas that have been cleared of tumors are indicated by a *.

doxycycline-regulatable version of oncogenic K-Ras, while at the same time expressing I-Sce1°. Then once tumors of a sufficient size had formed we would remove the doxycycline, oncogenic K-Ras would be turned off, and the tumors would regress. We hoped that if *Egfr* amplification had occurred in a small number of cells in the K-Ras driven tumor, that amplified mutant *Egfr* could compensate for the loss of oncogenic K-Ras expression, and the cells would be able to continue to grow and a tumor would form.

Rather than breed in a *tet-regulatable K-Ras^{G12D}* allele (Fisher et al., 2001) we decided to try to deliver oncogenic K-Ras on a lentiviral vector. We generated three different types of lentiviral vectors and confirmed their K-Ras induction, I-Sce1° expression, and Cre expression where applicable in cell culture (Figures 8B, 8C and data not shown) but only the simplest version, a tre-K-Ras^{G12D}-Pgk-Cre vector, gave reproducible tumor induction in a short timeframe and was used for all subsequent experiments (Figure 8D). We carried out all experiments on an *EIP; Xrcc4$^{FL/FL}$; CCSP-rtTA* or *p53$^{FL/FL}$; Xrcc4$^{FL/FL}$; CCSP-rtTA* background. We infected mice with the tet-K-Ras^{G12D}-Pgk-Cre lentivirus and administered doxycycline in the diet from day one. We monitored for tumor induction by microCT, and 8-14 weeks post-infection the mice were removed from the doxycycline diet. Tumors were found to regress by microCT (Figure 8E and data not shown), and the mice were aged for 6-9 months until the tumors re-emerged as monitored by either microCT or morbidity, and confirmed by histology. The tet-K-Ras^{G12D} lentiviral system displayed minimal leakiness based on the long-term survival of mice that received the virus in the absence of a doxycycline diet. However, we did find that tumors also recurred in the control background, *p53$^{FL/FL}$; Xrcc4$^{FL/FL}$; CCSP-rtTA*, but we currently don't have enough numbers to determine if there is a significant difference in the recurrence rate when *EIP* is included, which might be indicative of mutant *Egfr* amplification in the experimental group. The number of tumors that recur are usually between 1-5 per mouse and we have established a bank of these tumors and are now preparing to analyze their *Egfr* DNA copy number using a taqman assay. If any of the tumors show evidence of amplification we will then use the

remaining tumor pieces and histology to look at Egfr mRNA and protein levels and to assess amplification by FISH, respectively. As an alternative we also generated cell lines from the doxycycline-induced tumors to assess whether we could perform the complementation experiment *in vitro* with the advantage of easily modifying I-Sce1^{o} expression levels. Unexpectedly, these cell lines took upwards of a month to become established and in contrast to the *in vivo* situation, upon removal of doxycycline they continued to proliferate (data not shown), precluding their use in further assays.

Discussion

During tumor development cells acquire an array of changes ranging from mutation, to amplification, deletion or translocation. Amplification and overexpression of oncogenes have often been bracketed together as the end result is thought to be similar. However, with the advent of novel technologies that produce genome-wide information on copy number changes in tandem with expression data, this simple assumption may warrant revision. Although a common feature of many human tumors, gene amplification is poorly understood in terms of the underlying mechanism of amplification (Beroukhim et al., 2010). In addition to the mechanism of amplification major questions remain as to when amplification occurs during tumor progression, the context in which this can happen, the role, if any, of co-amplified genes in the amplicon, and whether tumors with amplification are similar to those with overexpression of the same oncogene.

The limiting factor that has hindered a deeper understanding of gene amplification and its relative contribution to tumorigenesis has been the absence of a system in which gene amplification can be induced to allow the process to be examined under a range of conditions. In this present study we set out to engineer a model of targeted gene amplification in the mouse. There is no precedent for this, and from the outset we had to base our strategy primarily on knowledge gleaned from models of amplification established in cell culture. We selected our conditional *Egfr*^{L858R} allele as a suitable candidate for targeted amplification since the mutation is coincident with amplification in 70-80% of cases, and mutation appears to precede amplification (Takano et al., 2005; Tang et al., 2005; Tang et al., 2008). We decided on a strategy that focused on the initiation of BFBs in lung epithelium and to this end, we adopted the yeast endonuclease, I-Sce1, as a tool to induce DSBs telomeric to *Egfr.* At the initiation of this project I-Sce1 was the only viable choice for this purpose, as the function of other enzymes in mammalian cells was poorly characterized, and zinc-finger nucleases were only beginning to emerge as a prospective tool for mammalian genome modification. While the contribution of

DSBs to amplification has been previously documented in mouse models of lymphoma (Difilippantonio et al., 2002; Zhu et al., 2002), there is no evidence that a DSB alone is sufficient to initiate gene amplification.

We have been unable at any point to satisfy all four of the criteria initially outlined as being essential to achieving gene amplification. The most significant barriers were the problems with achieving sufficient I-Sce1^{o} expression, and accordingly a sufficient frequency of I-Sce1^{o}-induced breaks, and the limited number of cells in S-phase at any given time in the lung. Our results on the ability of I-Sce1^{o} to induce the loss of the hygromycin cassette *in vivo* revealed that, despite low levels of expression as detected by western blot, we were actually able to express a sufficient amount of I-Sce1^{o} to induce two concurrent breaks on chromosome 11. The major obstacle is that there is no obvious way to design an allele that could give an accurate read-out of the number of breaks that have been induced so that we could have a measure of the overall frequency of the DSBs at a given concentration of I-Sce1^{o}. The other major hurdle that this approach presented was the low mitotic index of the lung with the result that even if we could induce large numbers of DSBs, only a very minor fraction would occur in a cell that was actively cycling, further reducing the probability of initiating a BFB cycle. We attempted to use naphthalene-induced lung injury and renewal to mitigate this issue, but again the low probability of a virally-infected cell that had recombined the requisite alleles, undergoing proliferation and a DSB in the same timeframe, diminished the impact this approach could have.

Our final strategy of an *in vivo* complementation approach, although completely artificial and distant from our initial goals, mirrors similar methods used in cell culture to identify mechanisms of therapeutic resistance (Rothenberg et al., 2008). Initial reports of tumors initiated using *tet-K-Ras*^{G12D} alleles in the lung found that these tumors regressed upon doxycycline withdrawal, even on the background of loss of *p53* (Fisher et al., 2001), but recent work has found that oncogene addiction is modulated in the presence of Mad2-induced genomic instability such that while tumors still regress in accordance with previous work, they recur at a

high frequency (Sotillo et al., 2010). Our data has revealed that in the context of the loss of both *p53* and *Xrcc4*, lentiviral induced K-Ras^{G12D} tumors eventually recur in a doxycycline-independent manner. Our method of delivery of K-Ras^{G12D} differs from the transgenic models and we have yet to determine if such recurrence occurs in the presence of *p53*, or even when both *p53* and *Xrcc4* are intact, as it is possible that the oncogene dependence of both models may not be similar. The significant time delay in tumor recurrence in our system is somewhat striking given the concurrent loss of both *p53* and *Xrcc4*, and raises concerns about the over-interpretation of the apparent oncogene addiction revealed by such doxycycline-inducible systems in the context of the level of oncogene-addiction that occurs in human tumors. These tumors arise very rapidly leaving little time to acquire secondary mutations, and this may not mirror the case of human tumor evolution.

The advent of tumor sequencing efforts and genome-wide copy number analysis has provided a wealth of information on the lesions that occur in human tumors, but a significant bottleneck is transitioning from this characterization to the point of determining whether these lesions are so-called 'driver' or merely 'passenger' mutations of minimal relevance (Beroukhim et al., 2010; Parmigiani et al., 2009; Weir et al., 2007). The lentiviral approach to oncogene delivery that we developed for the *in vivo* complementation experiment is a strategy that could be utilized to test the functional significance of newly discovered mutations in lung cancer, both in the context of initiating mutations, but also this vector could be combined with our *LSL-K-Ras^{G12D}* model of NSCLC to examine their role in tumor progression and metastasis.

One explanation of our inability to elicit gene amplification is that it failed simply due to technical hurdles, but an equally plausible interpretation of our negative results is that we currently have incomplete knowledge as to the critical factors that need to converge for amplification to be initiated. The possibility exists that DSBs are indeed sufficient as an initiating factor, but that they need to occur in certain chromatin contexts or in particular genomic locations to have the desired effect. Future efforts designed to not only identify, but also to

characterize, the nature of amplicons found in human tumors may elucidate important genomic features associated with amplification. Incontrovertible evidence supporting a role for DSBs in initiating amplification events has only been found in models of B-cell lymphoma in the mouse (Difilippantonio et al., 2002; Zhu et al., 2002). Cells of the lymphoid lineage undergo DSBs in the context of V(D)J recombination, and it is possible that as a result, the consequence of any induced DSB break are different in these cells compared to those in the lung, where such breaks don't typically occur. A more prudent, although less relevant, approach may have been to try to engineer *Myc* amplification in B-cells. This is a lesion known to occur in both mouse and human B-cell lymphomas, and the requirements for loss of DNA repair machinery and the initiating Rag2 DSB lesion are well documented, and may be an appropriate platform to initiate future efforts at engineering inducible gene amplification in the mouse.

In a manner reminiscent of how the *LSL-K-Ras*^{G12D} model has highlighted key differences between the signaling and phenotype that results from the activation of a single copy of oncogenic *K-Ras* compared with the overexpression of the protein, the establishment of a method to induce amplification in the mouse would no doubt enlighten us, both as to the role of amplification in tumor progression and the therapeutic implications of the differences between amplification and overexpression as a means to increase EGFR protein levels. To make assumptions as to the equivalence of such different oncogenic lesions based on an end-phenotype such as tumor formation, or sensitivity to TKIs, is at best a perilous path to follow, and typically there are many subtle, but often important, differences between lesions that can have tremendous biological and clinical significance. The true merit of attempting to accurately mimic human tumor evolution in the mouse is currently unknown, but only a deep understanding as to the molecular features underlying the progression from a single initiating lesion to an advanced, metastatic tumor will reveal the fragile components of the tumor network that should be aggressively targeted to lead to a robust, long-term therapeutic response.

Materials and Methods

Generation of I-Sce1-LHL-I-Sce1 mouse. BAC clone RP23-36D23 was ordered and targeting arms amplified with KL514/KL515 and KL516/KL517 primer sequences as listed. These were cloned into pKS-DTA and sequence verified. A floxed hygromycin cassette was amplified with flanking I-Sce1 sites using KL495/KL496 digested with AvrII and cloned into the compatible Xba digested vector. Note: the loxP sites in this construct are incompatible with those in the LSL cassette of the *Egfr* allele. Positive clones were sequence verified. v26.2 C57BL/6J ES cells were targeted with the linearized construct and selected in hygromycin (150µg/ml). Southern blotting of ES cells: DNA was digested with BamH1 for the 5'end and Kpn1 for the 3'end. PCR primers for genotyping the 5'end of the allele are Forw: 5'-GTCAGAGTTGTGACTGTGGG-3', Rev: 5'-CTAAAGCGCATGCTCCAGAC-3', and for the 3'end of the allele they are Forw: 5'-GTACTCGCCGATAGTGGAAAC-3', and Rev: 5'-CTAATGTGCCCAAGTGAGGAC-3'.

Generation of the ColA1-tetO-I-Sce1^{o} mouse. The cDNA for HA-I-Sce1^{o}, including a Kozak sequence, was cloned into the pBS31-tetO-pgkATGFRT vector obtained from the Jaenisch lab and sequenced verified. C10 ES cells (F1: C57BL/6J and 129Sv/Jae) containing the FRT-flanked neo cassette and promoterless hygro at the ColA1 locus were targeted with this construct and hygromycin resistant clones selected and expanded. A southern blot on Spe1 digested clone DNA was carried out, and probed with the 850bp '3'probe' digested out of the 3'probe plasmid obtained from the Jaenisch lab.

Lentivirus and retrovirus preparation. Retrovirus was prepared by combining a 1:1 ratio of retroviral vector:pCL-Eco with TransIT-LT1 transfection reagent (MirusBio), followed by transfection of 293FT cells (Invitrogen). For lentivirus preparation, 293FT cells were plated and 24hrs later, with the cells at 50% confluency, they were transfected with a 4:3:1 mix of

vector:delta8.2:VSVg combined with TransIT-LT1 transfection reagent (MirusBio). In both cases viral supernatant was harvested and filtered at 48hrs and 72hrs post-transfection, and added directly to cells in combination with polybrene (10μg/ml). Where applicable selection was initiated 24hrs after the final addition of virus, and cells were maintained in selection for 48-72hrs for puromycin (2μg/ml) (Sigma), or until all uninfected cells were killed. For *in vivo* experiments lentivirus was prepared using 15cm dishes of 293FT cells and the 48hr and 72hr supernatants were pooled, and ultracentrifuged for 2hrs, at 4°C and 25,000 rpm. The viral pellet from each 15cm plate was resuspended in 200μl of 1X HBSS, pH 7.4 and titered for Cre expression as previously described (DuPage et al., 2009). Virus that was to be administered within 4-5 days was stored at 4°C, otherwise it was aliquoted and stored at -80°C until needed.

Protein extraction and immunoblots. Cells were always lysed on ice following two washes with ice-cold PBS. The extraction buffer used was as follows: 10% Triton, 50mM $Tris_{7.4}$, 150mM NaCl, 0.5% sodium-deoxycholate, 0.1% SDS, 1mM DTT, 1mM EDTA, supplemented with Mini-protease inhibitor tablets (Roche) and either Phos-stop (Roche) or phosphatase inhibitor cocktails 1 and 2 (Sigma). Lysates were rocked at 4°C for 15mins followed by a 14,000rpm spin, 15mins, 4°C. Tissues were processed in a similar manner on ice, using either a razor blade or a small dounce to complete homogenization. Supernatants were quantitated using the BCA assay (Pierce), and diluted with a standard sample buffer containing β-mercaptoethanol. Lysates were run on SDS-PAGE gels, made in house or purchased from Invitrogen, and transferred to PVDF membrane. Antibodies used included: HA (Covance, MMS101-P, PRB-101P), β-tubulin (Sigma T4026, CST #2128), Egfr (sc-03, Santacruz).

Soft-agar assays. A 1:1 mix of 1.6% agarose (Seaplaque) and 2X media (Invitrogen) was made and 1.5ml was added to each 35mm dish and allowed to set at RT for 30mins. A 1:2:1

mix of 2X media: 1X media: 1.6% agarose was made and aliquoted with an appropriate number of cells, and where appropriate EGF, TGF-α, or AR, to a final concentration of 10ng/ml was added. The volume of cells and ligand added were removed from the volume of 1X media to preserve the correct total volume. 1ml of this mixture was then added to each plate and they were left to set for 1hr at RT. Plates were incubated at 37°C for 3 weeks, and a few drops of media, containing ligand where appropriate, were added to the plates each week to prevent them from drying out. For the actual screen 10cm dishes were used and volumes were scaled up accordingly.

RNA extraction, cDNA preparation and qPCR. RNA was isolated using either the Qiashredder and RNeasy Mini Kit (Qiagen) or with a standard Trizol extraction protocol, followed by a DNase treatment to remove any contaminating genomic DNA. cDNA was generated using 1µg of RNA with the Superscript III kit (Invitrogen) and random hexamers. qPCR was carried out with SYBR GREEN using an ABI PRISM 7000 Sequence Detection System Thermo Cycler (Applied Biosystems).

I-Sce1 expression and lentiviral vectors. HA-I-Sce1 was a kind gift from M. Jasin. Codon optimization of I-Sce1^{o} was carried out by GeneArt (Germany). Ubc-I-Sce1-PgK-Cre lentiviral vector was constructed from Ubc-Luc-Pgk-Cre (DuPage et al., 2011), and tre-I-Sce1^{o}-pGK-Cre and tre-I-Sce1^{o}-Ubc-rtTA-ires-blasticidin vectors were constructed in collaboration with J. Doench and E. Calo.

PCR analysis of DSB events. I-Sce1 enzyme was purchased from NEB. Primer sequences to assess loss of the hygromycin cassette are: Fow: 5'-AGAGTCTCTCACCTCCTAGGTA-3' and Rev: 5'-CACGGAGAAAGGAGATATCACC-3' and the incorporation of this third primer: 5'-GAGATCAGCAGCCTCTGTTC-3' which will give a band with the Rev primer listed above is

used to detect the intact LHL cassette. The expected size of the bands are: I-Sce-1-LHL-I-Sce-1: 266bp; I-Sce1-lox-I-Sce1: 241bp; I-Sce1 due to cutting: 160bp.

microCT analysis. Mice were scanned while under isoflurane anesthesia using a small animal eXplore Locus microCT (GE Healthcare). Images were acquired and reconstructed using GE eXplore Scan control software.

Mice. *Trp53*$^{FL/FL}$ mice were provided by A. Berns (Netherlands Cancer Institute), *Xrcc4*$^{FL/FL}$ mice were provided by F. Alt (Harvard Medical School, Boston, MA), *R26rtTA* mice were provided by R. Jaenisch (Whitehead Institue, Cambridge, MA), the *Villin-Cre* mice were obtained from the MMHCC, and the *CCSP-rtTA* mice were purchased from The Jackson laboratories. *Egfr*^{L858R}, *Egfr*$^{LSL-L858R}$, *K-Ras*$^{LSL-G12D}$ strains were generated in the Jacks lab. Adenoviral and lentiviral intratracheal infections was carried out as previously described (DuPage et al., 2009). Adenoviral cre tail-vein injection was carried out using 10^9 pfu of virus. Naphthalene was administered as either a single dose of 250mg/kg or as 4-5 lower doses of 100mg/kg over the course of 4-5 weeks. Carbontetrachloride (Sigma) was dissolved in corn oil and administered intraperitoneally at a dose of 3ml/kg of a 50% solution. Doxycycline diet of 625ppm was purchased from Harlan-Teklad. Animal studies were approved by the Massachusetts Institute of Technology's Committee for Animal Care and conducted in compliance with Animal Welfare Act Regulations and other federal statutes relating to animals and experiments involving animals, and adheres to the principles set forth in the 1996 National Research Council Guide for Care and Use of Laboratory animals (institutional animal welfare assurance number, A-3125-01).

Cell culture. MEFs were generated from E13.5 embryos and cultured in DMEM (DME, 10% FBS, 2mM glutamine, 1X Pen/Strep). mEGF, TGF-α, 4-OHT, and doxycycline were purchased from Sigma, AR from R&D Systems . HeLa Tet-ON cells were from Clontech.

Immunofluorescence. Cells were transfected with a UbcrtTA expressing plasmid, plated on coverslips, and mock treated or induced with doxycycline. Cells were then fixed in 3% PFA, permeabilized with 0.5%Triton, and blocked in normal goat serum. After an overnight incubation at 4°C with an antibody against HA, coverslips were washed and incubated for 45mins with an AlexaFluor 488 donkey anti-mouse secondary antibody (Invitrogen), and after addition of DAPI coverslips were mounted and imaged using an Axioplan2 (Zeiss) microscope using Openlab software (Improvision).

shRNA design and cloning. All hairpin target sequences were designed using pSicoOligomaker v1.5 designed by A.Ventura (MSKCC), and the corresponding oligos were then cloned into pSicoR-CMV-GFP. All cloned hairpins were sequence verified. Hairpin sequences are available upon request.

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Chapter 5

Discussion, Future directions, and Challenges

Overview

The ability of oncogenes to hyperactivate tumor promoting signaling pathways is often thought of as an unrestrained advance towards tumor progression, with only the oncogene-induced tumor suppressor network acting to constrain this signaling, and limit tumor induction. The origins of this thesis lie in the original discovery of EGFR kinase domain mutations in NSCLC patients who responded dramatically to treatment with gefitinib or erlotinib (Lynch et al., 2004; Paez et al., 2004). We decided to engineer a mouse model of one such mutation, L858R, with the goal of enhancing our limited, at least at the time, knowledge of the oncogenic nature of these mutations, along with an aspiration to construct a model that would facilitate an investigation of the molecular mechanisms that underlie the inevitable therapeutic resistance that emerges with such targeted therapies.

The unequivocal dependence of human mutant tumors on sustained EGFR signaling, and the rapid induction of tumors in transgenic models that were generated (Ji et al., 2006; Politi et al., 2006) left the field in no doubt as to the ability of mutant EGFR to drive tumor formation in the lung. The striking absence of tumors in our knock-in model, and the emergence of human data cataloging the common co-occurrence of mutations with gene amplification (Nomura et al., 2007; Soh et al., 2009; Takano et al., 2005) could have led us to conclude that a threshold level of EGFR protein is needed for tumor formation and that the levels produced from our allele simply lie below that. The decision to model our *Egfr* allele closely on the *LSL-K-Ras*^{G12D} allele previously generated in the lab led to a two-step knock-in approach, which fortuitously led us to generate germline mutant mice that did not contain a LSL cassette. A careful investigation using tissues and MEFs from these mice led us to the observation upon which almost this entire thesis is founded; namely, that mutant Egfr was expressed at lower levels than wild-type, and that this downregulation of the mutant receptor was dependent on Egfr kinase activity (Chapter 2). This re-routed our goals, and led us to focus on trying to understand the early signaling

events that occur upon expression of mutant Egfr, and the nature of the feedback loop that leads to a reduction in expression levels (Chapter 3). This alone didn't seem challenging enough, and we thought our L858R allele had in fact presented us with the opportunity to engineer a lesion that has so far evaded traditional mouse modeling approaches, gene amplification, and our attempts at modeling this are described in Chapter 4.

Negative regulation of oncogenic signaling

The involvement of negative feedback loops in restraining oncogenic signaling is neither novel, nor specific, to mutant Egfr. In the *LSL-K-Ras*^{G12D} model it was immediately noticed that the tumors that developed did not appear to hyperactivate the canonical effector pathways, p-Erk and p-Akt; furthermore, even though *lox-K-Ras*^{G12D} MEFs were partially transformed there was a muted response to serum stimulation and a decrease in steady-state levels of p-Erk and p-Akt compared to wild-type controls (Jackson et al., 2001; Tuveson et al., 2004). Although Spry2 has emerged as a potential regulator of the levels of p-Erk in these tumors (Junttila et al., 2010; Shaw et al., 2007), a complete understanding of how oncogenic K-Ras manages to promote tumor induction and progression in the presence of such feedback still has to emerge; furthermore, the question of whether such feedback is essential in the early stages of tumor formation, perhaps to constrain oncogenic signaling below a threshold required to trigger oncogene-induced senescence, remains unresolved.

Negative feedback regulation no doubt occurs in other tumor models; indeed, as mentioned it has been observed in the *LSL-K-Ras*^{G12D} model, and based on the existence of feedback in mutant cell lines is also a likely, though so far undocumented, feature of the *B-Raf*^{V600E} models (Pratilas et al., 2009). Negative feedback regulation that acts in tumors has probably evolved, not to specifically restrict tumor formation or progression, but to control signaling during normal cellular homeostasis. The level of feedback control directed at a protein probably depends on the position of the protein in a signaling pathway, the propensity for

aberrant signaling, and the downstream implications of any such activity. EGFR lies at the apex of a signaling pathway, acting as a conduit between extracellular signals and intracellular responses. In parallel with an expanding role for EGFR and an increase in the number of ERBB family members, a myriad of proteins have been co-opted to control and terminate signaling through the receptor after an appropriate response time has elapsed. Due to the potency and diversity of the signaling that emanates from the receptor, cells have a number of further fail-safe mechanisms in place in case of aberrant receptor signaling, for instance in a case where receptors remain on the surface for longer than normal. In contrast, both K-RAS and B-RAF function further downstream in the pathway, and under normal conditions their activation is typically directly controlled by elements that lie upstream, such as RTKs. As a consequence, the degree to which feedback control directly impinges on activated RAS or B-RAF is likely to be significantly less, as cellular control mechanisms might operate on the assumption that signaling from RAS came via an RTK, and hence that would be a target for suppression. From an evolutionary vantage point it makes sense why negative feedback would directly target EGFR, while in the case of RAS or B-RAF, signaling elements such as ERK and MEK are the preferred targets. An examination of the status of Egfr levels or activity in K-Ras^{G12D} lung tumors might be worth examining, and may lead to some insights as to the feedback mechanisms that are actively engaged in these tumors.

Tissue-specific effects of Mig-6 depletion

We initially decided to investigate whether altering the levels of any of the handful of known negative regulators of EGFR, could reverse the downregulation of the receptor. shRNAs against a number of these proteins failed to alter receptor levels in MEFs, so instead we utilized a conditional knockout allele of *Mig-6* (Jin et al., 2007). We reasoned that the ratio of receptor to negative regulator may be critical, and that complete elimination of a feedback inhibitor may be

required to elicit a noticeable effect. *Mig-6* deletion in MEFs extended Egf-stimulated signaling in both heterozygous and homozygous mutant MEFs, but failed to alter Egfr protein levels.

The consequences of *Mig-6* deletion *in vivo* were found to be both completely dependent on the presence of the mutant receptor and tissue-specific. No visible phenotype emerged in the lung, but benign papillomas developed on hairless regions of the body, including the nose, ears and tail. This tissue-specific phenotype of *Mig-6* deletion in the context of mutant Egfr raises some interesting questions for further investigation. The contrasting phenotypic consequences in the skin and lung upon acute loss of *Mig-6* in the context of mutant Egfr could be explained by one of the following models: (i) it is possible that Mig-6 only interacts with mutant Egfr in the skin and not the lung, causing its deletion to elicit a skin-specific phenotype, or (ii) *Mig-6* deletion unleashes mutant Egfr signaling in both tissues, but only in the skin is that signaling above a threshold sufficient to elicit a phenotype, or (iii) Mig-6 binds and actively suppresses mutant Egfr signaling in both cases, but mutant Egfr may require an additional external signal to fully initiate aberrant signaling. The validity of the first two models could be examined by carrying out a simple co-IP experiment in both tissues to look for interaction, together with monitoring activation of Egfr and downstream signaling components by IHC in a time course after tamoxifen addition to $Egfr^{LSL\text{-}L858R/+}$; $Mig6^{FL/FL}$; $R26^{CreER/CreER}$ mice. We favor neither of the first two models as EGFR and MIG-6 have previously been shown to interact upon ectopic expression of the mutant receptor in lung cells known as HBECs (Guha et al., 2008), and the frequency of EGFR mutations in NSCLC relative to other tumor types may suggest that the lung epithelium actually has a lower threshold in relation to the amount of EGFR signaling required to effect a phenotype. The third model is the one we favor, and since we only observed a phenotype upon *Mig-6* deletion in exposed areas of skin that are prone to wounding or other damage, including the tagged ear, we hypothesize that this secondary signal may be essential to result in complete activation of the newly uninhibited receptor. This concurs with our data from MEFs where the primary effect of Mig-6 deletion is not to increase, but to extend EGF-

induced signaling in the later stages of the time course. This result, together with an absence of any increase in steady-state signaling suggest that even in the absence of Mig-6, mutant Egfr requires some additional external signal before aberrant signaling is manifest. This model could be explored further by examining the consequences of combined *Mig-6* deletion and $Egfr^{L^{SL\text{-}858R}}$ activation, in the presence or absence of airway damage, for instance using naphthalene or bleomycin (Reynolds et al., 2000; Reynolds et al., 2007).

The *Mig-6* deletion experiments confirm that in certain contexts this negative regulator actively suppresses signaling from mutant Egfr, and that when deleted, the increased activity of the mutant receptor is unmasked. Recent data on the role of Mig-6 in GBMs revealed that while Mig-6 can alter signaling in EGFRvIII expressing cells, that it appears to do so by regulating activity or availability of the wild-type receptor and not of EGFRvIII (Ying et al., 2010). Our results with $Egfr^{L^{858R}}$ reveal a signaling and/or phenotypic effect upon *Mig-6* deletion in both L858R hetero- and homozygous MEFs and mice, although the relative contribution of the wild-type receptor has not been assessed. These mutations, EGFRvIII and L858R, while both activating, are structurally different, are typically found in different contexts, brain and lung respectively, and likely engage different downstream signaling responses. Any of these factors could contribute to the differential sensitivity of these mutants to *Mig-6* loss. Finally, as more negative regulators are being actively characterized, many like MIG-6, are shown to have important roles in regulating signaling during development, and as such their deletion can have pleiotropic effects. As exemplified by the contrasting phenotypes upon germline and somatic deletion of *Mig-6* in the lung, a concerted effort should be made to assess the role of these negative regulators in tumor formation primarily through the use of either conditional knockout alleles or conditional shRNA technology, to avoid confounding results due to developmental defects.

ERK and CULLIN5 regulation of mutant Egfr

Our principal finding is that mutant Egfr is maintained at lower levels than the wild-type receptor due to the induction of negative feedback mechanisms. Based on this we speculate that this downregulation is in fact the selective pressure that leads to the amplification of mutant EGFR found in many of these lung tumors. Amplification may simply act to overcome the feedback mechanisms and re-establish wild-type levels of the mutant receptor. If samples from the appropriate human tumors were available it would be interesting to compare the relative protein expression of EGFR in tumors with amplified and non-amplified mutant *EGFR*. It is possible that the steady-state levels are the same or at least that the fraction of active receptor is similar, although it is probably equally likely that due to the nature of the lesion and the mechanisms through which amplification is thought to be established that the levels of gene amplification far exceed those required to actually form a tumor, leading to high level expression.

Our results initially left us in a quandary as to how to explain the incidence of human tumors harboring EGFR mutations without gene amplification. We hypothesized that some of our proposed negative regulators might be deleted or functionally disabled in these tumors releasing the mutant receptor to hyperactivate downstream signaling pathways. Equally perplexing was that we could find no evidence in either L858R MEFs or tissues of differential induction of signaling pathways or any substantial induction of known negative regulators. The decreased expression of the mutant receptor was almost the only evidence that a mutation had been engineered into these cells. We proposed a model where expression of the mutant receptor induces a burst of oncogenic signaling, that is propagated into negative feedback pathways that act directly at the level of the receptor. Over time a balance between active receptor signaling and negative feedback is established; the feedback maintains the receptor at low enough levels such that the signaling emanating from the receptor is grossly equivalent to wild-type, and the steady state is composed of low signaling output from the receptor and a corresponding low induction of feedback pathways. It was this steady-state situation that we

believed was represented in the tissues and MEFs of germline mutant mice and this was our explanation for our inability to find any evidence for feedback in our mutant Egfr expressing cells.

A system was required that would allow us to examine mutant Egfr signaling shortly after the receptor was expressed. Using the inducible 293 T-REx FLP-in system we were able to recreate the downregulation, and found that it occurred with rapid kinetics, within 48hrs of induction. This downregulation showed similar features to what we had observed in MEFs, *i.e.* a dependence on Egfr kinase activity and the involvement of the proteasome in receptor degradation. We have gone on to use this system to identify the ERK signaling pathway and the CULLIN5 E3 ligase as key determinants of this downregulation. The exact role of ERK pathway signaling in Egfr downregulation is currently unclear, but from recent data it appears to be connected to ERK-dependent phosphorylation of Egfr at Thr-693. The proposed next steps are to assess whether Thr693 is aberrantly phosphorylated in any of the human NSCLC cell lines harboring EGFR mutations, and if so, whether this phosphorylation is responsible for the reduced internalization rate of the mutant receptor. We need to assess the localization and trafficking of the mutant receptor and whether it might be altered in the presence of U0126 or in the context of Dusp6shRNAs, as this may allow us to connect alterations in receptor localization to recruitment of CULLIN5. As mentioned previously, ERK and CULLIN5 may be acting independently on the mutant receptor or they may be connected and this is an area that needs to be clarified. One approach to this would be to assess CULLIN5 association with mutant Egfr, either by IF or by co-immunoprecipitation, in these cells and monitor whether modulating ERK pathway components affects this interaction. Although we have managed to co-immunoprecipitate the receptor with CUL5, this has only been done in the context of transient transfection, and it is still possible that CUL5 is not acting directly on Egfr. While this 293 T-REx system has been useful for assessing the role of possible candidates in mutant Egfr degradation, what is currently lacking is data showing that modulating some of these candidates in another system can both alter Egfr levels and critically either boost signaling, or elicit some phenotypic

response, anywhere from increased proliferation to growth in soft agar. As detailed in Chapter 3, experiments are underway to address this using a NIH-3T3 system expressing mutant Egfr and also by attempting to knockdown Cul5 in the lung using lentiviruses expressing shRNAs.

Our expectation is that some components of this negative feedback loop may be deleted or functionally inactivated in human tumors that carry mutation but no amplification. These tumors should be screened to see if such deletions occur, and if found it would solidify this as an alternative mechanism to amplification through which mutant EGFR expressing cells can attain threshold levels of signaling and become oncogenic. CULLIN5 and ERK were selected based on literature searches, known pathways that couple to EGFR, and the fortuitous availability of pathway inhibitors. An unbiased approach would no doubt identify other candidates involved in the feedback, and would perhaps help us to connect ERK to CUL5, if indeed such a connection exists. Due to the rapid degradation of mutant Egfr by 48hrs in the 293 T-Rex cells we have a robust phenotype that might be amenable to a screen. The lines could be re-made with a GFP-tagged version of mutant Egfr and once the downregulation has been confirmed in these lines, either a genome-wide shRNA or cDNA screen could be conducted with the output being to simply sort for cells expressing an increased level of GFP at 48hrs.

Finally, the rapid downregulation of mutant Egfr observed in the T-REx system should motivate us to try and capture early signaling events from endogenously expressed *Egfr-L858R*. One approach would be to look immediately after the first signs of Cre or Cre-ER mediated recombination in either MEFs or tissues. Alternatively, Egfr signaling could be inhibited using a TKI such as erlotinib, and the reactivation of downstream signaling pathways could be monitored after the drug is washed out. As long as cells are analyzed very rapidly after receptor expression, either approach should enable the identification of some elements of aberrant signaling or feedback. It would also be worth taking a look in our *K-Ras* model to see if there is a similar pulse of early signaling that is then dampened over time. Such strong feedback

mechanisms are probably a universal feature of how cells respond to an oncogenic mutation, but detecting them, and understanding the early responses to oncogene activation are significant challenges.

Threshold levels of oncogene expression

Apart from mouse models of c-Kit mutation, which have been identified in both somatic and germline cases of GIST (Rubin et al., 2005; Sommer et al., 2003), the majority of tumor mouse models that involve an RTK have utilized transgenic rather than knock-in approaches, although this is primarily because many of these RTKs are overexpressed rather than mutated in human tumors. EGFRvIII is an interesting exception in that, in an analogous manner to EGFR-L858R, it is both mutated and amplified in tumors (GBMs), and while transgenic expression does result in tumor formation, the question of whether this mutant receptor is also susceptible to negative feedback remains. In lieu of constructing a mouse model that mimics the deletion at the endogenous locus, we would suggest constructing a 293 T-REx line that expresses EgfrvIII in an inducible manner, and monitor whether it too is degraded faster than the wild-type. If this is indeed found to be the case then a comparative analysis between EgfrvIII and Egfr-L858R expressing cells, for instance using phosphoproteomics or microarrays, may actually be a useful approach to try and tease out the signaling pathways involved in the degradation from the background of signaling pathways induced by the activated receptor.

Our *Egfr-L858R* allele failed to induce the tumors we had hoped for, and reinforces the idea that threshold levels of signaling, mediated either through hyperactive proteins or increased levels of these proteins, are required for tumor formation. It also suggests that a high level of oncogenic signaling may not be advantageous if it elicits a correspondingly high level of feedback; accordingly, a more astute approach may be to increase signaling but remain below a threshold needed to engage high-level feedback, such that the balance favors the forward signaling. Our results raise the interesting question of how cells distinguish low level oncogenic

signaling from normal signaling, if indeed they even can. Mutant Egfr signaling is somehow being recognized as abnormal and this allele could be used to further our understanding of the triggering mechanism involved, and perhaps how oncogenic K-Ras, either fails to initiate such signaling or evades recognition by feedback proteins. Once again this requires tools to rapidly analyze signaling pathways in the immediate aftermath of a point mutation occurring. Rather than trying to develop ways to express Cre more efficiently, we would suggest that one approach to this would be to infect target cells with a tet-regulatable hairpin against Egfr and induce Cre-mediated recombination of the LSL cassette in the presence of doxycycline. Once a pure population of recombined cells is established the hairpin would then be shut off by withdrawal of doxycycline and mutant Egfr would be expressed. The advantage is that this approach would be amenable to many oncogenes, although, the timing may still be an issue depending on the turnover of the hairpins. This idea is modeled on the recent report from the Lowe lab using tet-inducible shRNAs for loss-of-function studies in the mouse (Premsrirut et al., 2011).

The contrasting results of our allele with those observed in the transgenic models highlight the importance of threshold levels of signaling in tumor initiation; however, the question remains as to whether such threshold requirements are maintained as tumors progress and acquire numerous other lesions. The transgenic models could be used to address whether oncogene addiction in the context of mutant EGFR changes over the course of tumor development, for instance upon loss of p53 or acquisition of metastatic potential. The results could have profound clinical implications; namely, if a certain threshold level rather than an 'all or none' effect remains in place as tumors progress, then complete inhibition of activity may not be required to see a therapeutic response. This question of thresholds and oncogene addiction could also be addressed by combining the transgenic models with our allele, to assess whether a single mutant copy is sufficient to prevent tumor regression upon withdrawal of doxycycline at different points during tumor progression.

Two facets of the distribution of EGFR mutations that remain to be fully explained are 1) why the mutations are predominantly restricted to lung tumors, and 2) why these mutations are more commonly found in East Asians, than for instance Caucasians. Most oncogenic mutations show a proclivity for particular tissues, and this could be due to the expression pattern of the gene; the signaling context, for instance whether other key components of the pathway are expressed such as ERBB3 in the case of mutant EGFR; the exposure to a carcinogen; the differential induction or expression of negative regulators; or it may be related to tissues having different requirements for an oncogenic lesion to initiate a tumor. In the lung oncogenic lesions, such as K-Ras^{G12D}, may fail to induce tumor suppressor pathways and so the barrier to tumor initiation may be lower than in other tissues that require a prior or coincident loss of the p53 pathway (Young and Jacks, 2010). The tet-EGFR^{L858R} transgenic models could be used to test this by analyzing signaling and tumor induction in different tissues upon doxycycline administration, with the caveat that the overexpression may negate some of the tissue-specific restrictions that would normally act to restrict EGFR signaling.

The frequency with which these mutations occur in NSCLC cases in East Asians is probably connected at some level to why these mutations are observed predominantly in lung cancer. To date no environmental cause has been linked to this bias in mutation incidence, and the broad spectrum of countries across East Asia where this trend has been found, along with the increased prevalence of EGFR mutations in Asians living in the U.S., suggests that the cause is more likely to be genetic in origin. Once sufficient data is gathered on whether this bias holds in Asians born in the U.S., a clear assessment of the contribution of an environmental component should be possible. Haplotype analysis has also failed to find any meaningful association between polymorphisms linked to EGFR and the incidence of EGFR mutations in East Asians; a recent study found that the EGFR polymorphisms commonly found in Asians were actually more likely to lead to decreased expression of the receptor (Nomura et al., 2007). One hypothesis that we have attempted to explore is that the threshold requirements for

oncogenic EGFR signaling in Asians are reduced such that less mutant receptor is required to produce a tumor phenotype. We hypothesized that this could be due to negative regulators of EGFR being either less efficient, or having reduced expression, in Asian populations. EGFR mutations might occur with equal frequency in Caucasians, but if they never progress to tumors due to increased suppression mechanisms then they will never be detected. We reasoned that mutant EGFR might undergo reduced downregulation upon ectopic expression in cells derived from Asian populations compared with those from Caucasians. We purchased a panel of fibroblast lines of different ethnicities, some wild-type, others with genetic abnormalities, and attempted to infect them with lentiviruses expressing mutant Egfr. To date, we have been unable to obtain reproducible expression of Egfr in these lines and that has thwarted our efforts to investigate this possibility further. If CULLIN5 or ERK pathway signaling are involved in mutant EGFR regulation in humans, then it would be worth looking for polymorphisms in these genes between Asian and non-Asian populations. A global analysis of the ERBB signaling network in different populations would be an important step towards understanding the high incidence of EGFR kinase domain mutations in the East Asian population.

Modeling gene amplification in the mouse.

The decision to attempt to engineer a mouse model of gene amplification was rooted in the belief that important differences would be gleaned from the ability to compare models in which high levels of mutant proteins were expressed from the outset, with those where tumors are initiated with normal levels of expression of an oncogene but with amplification occurring gradually over the course of tumor evolution. Our labored efforts to induce *Egfr* gene amplification, both in the mouse and in cell culture, have ultimately failed, but through the course of these experiments we have gained a deeper understanding of the challenges faced in constructing such a model, and the key criteria that must be met for any future such approach to succeed. Unlike other genetic lesions that have been successfully modeled in the mouse,

constructing a mouse model of gene amplification is accompanied by the added burden that the underlying mechanism responsible for gene amplification in tumors is not well understood. A number of modifications to our system that we envisage would increase the probability of success are detailed below.

Detection.

Our decision to focus our amplification approach on $Egfr^{L858R}$ in the lung was based on the obvious relevance to human NSCLC, and on data from both transgenic models (Ji et al., 2006; Politi et al., 2006) and lentiviral expression of mutant Egfr (Chapter 2) which suggested that tumors would develop upon the expression of a sufficient level of mutant Egfr in the lung. However, the threshold level of Egfr expression required for lung tumor formation is not known. This represents a critical flaw in our approach, as the system we designed will only give a read-out, *i.e.* a tumor, if sufficient amplification has occurred. We cannot distinguish between a complete failure to induce amplification and a situation where amplification has occurred but levels of the receptor remain below a threshold needed for tumor formation. We originally considered including a reporter in our system but to simplify matters eliminated it from the final construct we proposed to make; it would be prudent for future attempts to include this element. The basic idea would be to include a Pgk-GFP cassette between the target gene and the DSB sites, either as a separate targeting construct or at the 5'end of the I-Sce1 cassette. If amplification does occur this could be monitored to some degree by an increase in GFP levels, in theory allowing single cells that have amplified the receptor to be identified in tissues, and importantly it would make a screen for amplification in cell culture, that is not dependent on selecting out a phenotype, feasible. A caveat with this approach is that DSBs are often processed which would potentially lead to deletion of the GFP cassette if it is located close to the break site, but this could be avoided if the GFP cassette is inserted closer to the gene that is the target of amplification.

Local chromatin structure and repeat elements.

The genomic context in which the targeted DSBs are induced probably contributes to the outcome. While a role for chromatin modification in the repair of breaks by NHEJ or HR pathways is well established (Hartlerode and Scully, 2009), as is the differential requirement for ATM in the repair of breaks occurring in heterochromatic or euchromatic regions (Goodarzi et al., 2008), relatively little is known about how, or if, the local chromatin structure might alter the frequency of amplification in response to a DSB.

Ideally a number of genomic locations would be assayed to determine the optimal site to maximize enzyme accessibility and cutting, and to minimize repair. At minimum a comparison of amplification frequency when sites are targeted to gene-rich or transcriptionally active regions with those targeted to gene-poor areas would be a suitable approach. In lieu of this, a systematic analysis of the borders of newly cataloged tumor amplicons (Beroukhim et al., 2010; Lockwood et al., 2008) may reveal general features of the common breakage sites, which might inform the decision as to where to insert I-Sce1 sites. The composition of the DNA adjacent to the cut site may have profound effects on the outcome; recent data from yeast supports a model whereby the presence of inverted repeats in close proximity to the DSB may facilitate the formation of hairpins through intrastrand annealing, leading to efficient palindrome generation, and the initiation of BFB cycles (Narayanan et al., 2006; Tanaka et al., 2005; Tanaka et al., 2007). The inclusion of short inverted repeat sequences also led to the efficient formation of palindromes and an increase in the frequency of gene amplification using an I-Sce1 based model of methotrexate-induced DHFR amplification (Tanaka et al., 2002). Future attempts at modeling amplification should incorporate these features and either include short inverted repeat sequences in close proximity to the break sites, or insert the restriction sites next to endogenous repeats or palindromic sequences.

Optimization of amplification conditions in cell culture.

Due to the multitude of factors that could impact the efficient induction of gene amplification it would be wise to set up a cell culture system that could determine the critical features that modify amplification rates for the gene of interest, and only once they have been validated to move into the mouse. The way I envisage this being done would be to generate a series of targeted ES cells all of which would contain a selection cassette, either DHFR or GFP, in combination with any number of variations on targeted location, presence of repeats, number of repeats, number of potential cut sites etc. The efficiency of amplification could then be assessed either by the frequency of methotrexate resistant clones or by using flow cytometry to assess GFP levels. Once the optimized conditions have been identified these features could be incorporated into the actual final targeting strategy.

Alternatives to I-Sce1.

The selection of I-Sce1 as the enzyme to induce DSBs was based on the frequent use of I-Sce1 in the DNA repair field to investigate HR, along with its use in cell culture models of gene amplification. While variants of I-Sce1 have emerged, including an ER-regulatable version shown to have improved nuclear localization (Hartlerode et al., 2011), a limitation of the use of any one particular enzyme is that a new mouse allele needs to be generated each time the user wants to modify either the location or number of DSB sites. The advent of zinc-finger nucleases has provided an alternative option whereby any location in the genome could be selected and a zinc-finger nuclease designed to induce a DSB targeted to that precise location. The relative efficiency of zinc-finger nucleases at inducing DSBs compared to endonucleases such as I-Sce1 remains to be characterized, but based on the efficiency with which they can generate correctly targeted human ES cell clones it might be feasible to use them for this process (Hockemeyer et al., 2009). If multiple zinc-finger proteins could be expressed in the same cell an array of DSBs could be induced at a series of genomic locations telomeric to the target gene, or DSBs could be induced at two or more independent loci in the same cell. This would provide,

at least in theory, the opportunity to test the effect of a limitless number of permutations concerning the position and number of DSB sites on the frequency of amplification.

The generation of a mouse model of targeted amplification is essential to understanding the contribution of this lesion to tumor formation and progression. Further efforts to develop such a model are essential, and when successful will no doubt improve our mechanistic understanding of how this lesion is generated in tumors, and how amplification can influence tumor progression and therapeutic response.

Challenges for the cancer mouse modeling field

A significant fraction of this thesis, for better or for worse, has been spent on the construction and characterization of alleles in the mouse, and as such the final words will be given to challenges and opportunities in the field of cancer mouse modeling.

The characterization of both human tumor samples and cell lines at almost every level imaginable, all on a genome-wide scale, together with the ability to carry out genome-wide shRNA and cDNA screens in these cells, has led to the sudden feasibility of many studies in human cell lines that were once the almost exclusive domain of the cancer mouse modeling field. Cancer mouse modeling has advanced far beyond a point of determining whether certain genes, or combinations thereof, are sufficient to induce tumorigenesis in specific tissues. Therefore, the goal of mouse modeling should not be to tackle questions that can be adequately addressed in cell culture experiments simply for the merit of carrying them out in the context of a whole organism; while there will occasionally be differences between the systems, the mouse is an expensive model system to use for verification purposes. Instead, together with using GEMs as pre-clinical models for testing therapeutics, the objectives of the field should be to address those questions that simply can't be explored in any other system. Such questions could include the interactions of tumors with the immune system or the microenvironment, tumor evolution from a signaling network perspective and with a focus on the functional interrogation of

mutations or alterations associated with different stages of tumor progression, and questions related to the cell-of-origin of tumors. Limitations are a feature of all model systems, from human cell lines to the mouse, and they should be considered when selecting the questions that the mouse is uniquely poised to address.

Below are what I believe to be some of the burgeoning challenges facing the ever-expanding cancer mouse modeling community, and potential approaches directed towards solving some of them. The solutions proposed for some of these challenges are generally too complex to actually work and merely represent some initial ideas on how to approach these problems. These all have as their end goal an improved understanding of basic tumor development, and as such do not address any of the myriad challenges accompanying efforts to use GEMs to develop novel therapeutic or diagnostic strategies. These particular challenges were also selected as they will require the development of novel, sophisticated tools, and will likely draw heavily from innovative methods under development in the field of synthetic biology; an area that has piqued my interest in recent years.

The four challenges:

1. As stated many times during this thesis, gene amplification or the overexpression of oncogenes occurs frequently during the progression of human tumors, but tools do not currently exist to adequately model this in the mouse. While modeling genomic amplification on the basis of BFB cycles is one approach, a method of dialing up, or down, the expression of a given gene at any point during tumor progression would be a hugely important advance. Currently the only strategy to do this involves the doxycycline-inducible system. Although, this has been successfully employed to examine the effects of low and high RAS expression in mammary tumors (Sarkisian et al., 2007), it is limited in that it typically functions at best in only three expression states: off, low or high, and in many cases it acts simply as an off/on switch, with a element of leakiness. We have recently tried to model in cell culture the transition from single

copy oncogenic K-Ras to the amplification found in many advanced mouse lung tumors. We employed a novel inducible system developed by the Collins lab, that is proposed to be tunable upon addition of different concentrations of IPTG and to display tight control over induction of expression (Deans et al., 2007) but for unknown reasons we have been unable to generate stable cell lines using this approach. This system, although complex in terms of the number of components, is the kind of approach needed in the mouse to allow the expression of cDNAs to be modulated at will and to a desired level. For instance, *LSL-K-Ras*^{G12D} could be activated in the lung as normal, and upon addition of an inducer at a given time, levels of oncogenic K-Ras could be slowly or rapidly increased, allowing the phenotypic consequences of such an alteration to be assessed under different conditions, such as the presence or absence of p53. Such a system could also be used to address whether levels of an oncogene influence oncogene addiction, and to define threshold levels of oncogene expression required in different tumor types.

2. Metastasis accounts for 90% of all cancer deaths yet attempts to study this component of the disease in autochthonous models have been beset by problems; specifically, their low frequency of occurrence and difficulty in detection, and mice dying from their primary tumor burden before metastases can be analyzed, treated or modified. A method to identify metastases, both local and distant, and tools to eliminate the primary tumor would allow this clinically relevant stage of the disease to be interrogated further. Current approaches to identify metastases involve labeling the primary tumor with a fluorescent protein, but the large signal from the primary tumor typically occludes the signal from any nearby metastases restricting this approach to the identification of distant metastases, at least in living animals. This issue could potentially be circumvented by identifying transcription factors whose expression is upregulated during metastatic progression; binding sites for these transcription factors could then be connected to a reporter such as GFP, and the induction of GFP expression could be used to identify metastases, or to sort cells in the primary tumor that display metastatic potential. With

some tweaks the same approach could be used with transcription factors that are downregulated upon metastatic progression.

If the primary tumor could be eliminated after a certain period of time this could be used to loosely mimic the situation upon surgical removal of human tumors. Due to the predicted increase in survival times this would allow metastases to develop further, and potentially afford the opportunity to induce secondary alterations in these metastases, or assess the response to therapy. Surgical removal of mouse tumors, especially in the case of the lung, remains a daunting prospect. An approach is needed that would express a molecule such as the diphtheria-toxin receptor (DTR) on the surface of lung tumor cells but only while they are resident in the lung; expression of the receptor could somehow be coupled to expression of some lung specific microenvironment factor. Upon exiting the lung, cells would no longer receive this signal and DTR would cease to be expressed. Upon later administration of diphtheria toxin (DT) the primary tumor would be eliminated but the metastases could continue to develop and progress. This idea is similar to the band-detect system that has recently been engineered in bacteria (Basu et al., 2005). Apart from allowing metastases to be studied independent of the primary tumor, such a system would allow the question of when metastases arise, and whether metastatic cells can remain dormant for extended periods of time to be formally addressed, as the primary tumor could be eliminated at any stage and the effect on the frequency of metastases monitored. The transplant of cells ectopically expressing tet-inducible oncogenes has been an initial step towards these goals (Podsypanina et al., 2008), but these experiments ideally should be done in the setting of autochthonous tumor models.

3. As the number of alleles generated increases a challenge facing the field is how to deal with incorporating multiple alleles into models, while also attempting to separate events temporally, in an effort to more closely recapitulate the multi-step model of tumorigenesis. The use of different recombinases is one approach to tackle this, but requires the generation of separate FRT'd and floxed alleles, tools to express Cre and Flp, and is eventually becomes limited by the

number of site-specific recombinase (SSR) systems available. Extending much beyond two to three genetic lesions that could be induced in a tumor at different times is not currently feasible, would involve large numbers of alleles, and this is without even considering any efforts to modify the microenvironment independently of the tumor. The recent development of doxycycline-regulated shRNA technology for use *in vivo* is a tremendous advance and adds another arm to the toolkit (Premsrirut et al., 2011), providing a rapid way to do loss-of-function studies in tumors. Incorporating multiple alleles that are activated by different recombinases or inducers also necessitates a system that allows event B to occur only in cells in which A was expressed, and C only where A and B were expressed *etc.* While the current approach of layering different systems together will work for two or three such events, it will quickly become unmanageable, and novel, faster approaches that are more modular are needed, especially if we wish to establish tumors using multiple alleles and then introduce a fourth or fifth lesion to test the functional contribution of a gene to tumor maintenance, metastatic potential, or therapeutic response.

4. Tools to monitor signaling dynamics at the single cell level *in vivo* are limited, and while human tumor samples have been analyzed with phospho-specific flow cytometry (Kotecha et al., 2008) this has not been widely used to study tumors from autochthonous models. The application of synthetic biology approaches to the design of sensors that could act as detectors for signaling pathways, such as for p-Erk or p-Akt activation, would allow these pathways to be monitored *in vivo* at the single cell, rather than the bulk tumor level. These tools would also produce a more quantitative readout of signaling through the pathway than is currently feasible using standard immunohistochemistry (IHC) approaches. Such synthetic biology driven approaches are needed as the current level of sophistication in designing mouse models is far in advance of our ability to adequately interrogate and classify the tumors that arise at a molecular level. The design of sufficiently sensitive *in vivo* biosensors will be challenging, but essential, to gain a complete understanding of the signaling dynamics at initiation, or of how

pathways are rewired upon tumor progression, along with monitoring therapeutic response. These approaches could also be tailored to monitoring the induction of tumor suppressor pathways or DNA damage responses in tumors, and it would be interesting to develop systems that could both present a readout of the current state of the pathway, but also record if the pathway had ever been engaged, and ideally how many times this had occurred, in essence giving a readout of the 'history' of the cell. This is similar to recent attempts to design synthetic counters (Friedland et al., 2009).

As a proponent of mouse modeling it has been sobering to realize how difficult it can be to effectively design even the simplest system and get it to work in cell culture, let alone in the mouse. A method to dial up Egfr expression in the presence of an inducer, resulting in three or four independent but reproducible expression levels, should be feasible, yet currently is not practical. A cursory glance at any organism will find that it is littered with examples of such circuits or systems that seem to function in a remarkably controlled and reproducible manner. While we may know the basic components of these systems we lack a complete understanding of the design features of these gene networks that are key to their function. An improved understanding of how natural systems function will have a tremendous impact on the design of the next generation of tools for interrogating mouse tumor models; tools that are essential if we are to maximize the impact that studies using mouse tumor models will have on our understanding of basic tumor biology.

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Presentations

K. Lane and T. Jacks. 'Modeling $EGFR^{L858R}$ NSCLC in the mouse'.

Poster, MIT Integrative Cancer Biology program retreat. December 2009.

K. Lane and T. Jacks. 'Modeling $EGFR^{L858R}$ gene amplification in NSCLC'.

Poster, AACR Mouse models of Cancer Conference, San Francisco, CA. January 2009.

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K. Lane 'Investigating the role of *Egfr* mutations in the mouse'

Talk, Colrain meeting, October 2005.

K. Lane and T. Jacks. 'Investigating the role of *EGFR* mutations in the mouse'.

Poster, CSHL symposium, Molecular approaches to controlling cancer. June 2005.

Teaching Experience

Spring 2006 TA for Introductory Biology

Spring 2004 TA for Cell Biology