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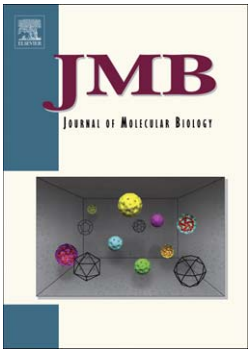

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Exploiting synthetic lethality and network biology to overcome EGFR inhibitor resistance in lung cancer

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Abstract

Abbreviations: ALK, Anaplastic lymphoma kinase; ALL, Adult lymphoblastic leukemia; CRISPR, Clustered regularly interspaced palindromic repeats; DAISY, Data-mining synthetic lethality identification pipeline; EGFR, Epidermal growth factor receptor; EGFRi, Epidermal growth factor receptor inhibitor; EMT, Epithelial-mesenchymal transition; GEMMs, Genetically engineered mouse models; GIIβ, Glucosidase II β-subunit; GRB2, Growth factor receptor-bound protein 2; GSEA, Gene set enrichment analysis; HBEC, Human bronchial epithelial cell; HER2, Human epidermal growth factor receptor 2; IGF-1R, Insulin-like growth factor receptor 1; IKK, IκB kinase; IL-6, Interleukin 6; K-Map, Kinase connectivity map; MAPK, mitogen-activated protein kinase; MK12, mitogen-activated protein kinase 12; NF-κB, Nuclear factor-kappa B; NK, Natural killer cells; NSCLC, Non-small cell lung cancer; ORR, Objective response rate; PD-1, Programmed cell death protein 1; PD-L1, Programmed death ligand; PFS, Progression free survival; Ph+, Philadelphia chromosome positive; RNAi, RNA interference; SCLC, Small cell lung cancer; SFK, Src family kinases; SHC1, SHC-transforming protein 1; shRNA, Short hairpin RNA; siRNA, Small interfering RNA; STAT3, Signal transducer and activator of transcription 3; SynLethDB, Synthetic lethal database; TAP-LC-MS/MS, Tandem affinity purification-liquid chromatography-mass spectrometry; TILs, Tumour infiltrating lymphocytes; TKI, Tyrosine kinase inhibitor; TNBC, Triple negative breast cancer; TNF, Tumour necrosis factor; TNKS1, Tankyrase 1; TNKS2, Tankyrase 2; TUSON, Tumour suppressor and oncogene; VHL, Von Hippel-Lindau tumour suppressor.

Despite the recent approval of third generation therapies, overcoming resistance to Epidermal Growth Factor Receptor (EGFR) inhibitors remains a major challenge in non-small cell lung cancer (NSCLC). Conceptually, synthetic lethality holds the promise of identifying non-intuitive targets for tackling both acquired and intrinsic resistance in this setting. However, translating these laboratory findings into effective clinical strategies continues to be elusive. Here we provide an overview of the synthetic lethal approaches that have been employed to study EGFR inhibitor resistance and review the oncogene and non-oncogene signalling mechanisms which have thus far been unveiled by synthetic lethality screens. We highlight the potential challenges associated with progressing these discoveries into the clinic including context dependency, signalling plasticity and tumour heterogeneity; and offer a perspective on emerging network biology and computational solutions to exploit these phenomena for cancer therapy and biomarker discovery. We conclude by presenting a number of tangible steps to bolster our understanding of fundamental synthetic lethality mechanisms and advance these findings beyond the confines of the laboratory.

Keywords: Non-small cell lung cancer; synthetic lethality; Epidermal Growth Factor Receptor; drug resistance; network biology; tumour heterogeneity; signal transduction.

Introduction

The discovery over a decade ago that non-small cell lung cancer (NSCLC) patients who harbour Epidermal Growth Factor Receptor (EGFR) mutations selectively respond to the EGFR inhibitors (EGFRi) gefitinib and erlotinib brought about an exciting era of personalised medicine in this class of difficult-to-treat cancers [1, 2]. These drugs have led to improvements in median Progression Free Survival (PFS) from 4.6 months to 13.1 months [3], which was unprecedented in lung cancer at the time of its discovery. Despite this success, the overwhelming majority of patients who initially respond to EGFRi therapy relapse within 16 months due to acquired drug resistance [4]. The most frequently observed and well-characterised mechanism of acquired resistance is the T790M gatekeeper mutation in EGFR [5]. In the past year, the approval of the T790M-selective inhibitor osimertinib for the treatment of this cohort of patients demonstrates how a deep understanding of the molecular mechanisms of acquired drug resistance facilitates the development of next generation therapies for overcoming resistance and delaying tumour recurrence [6]. However, recent clinical data suggests that third-generation inhibitors will be similarly challenged by the emergence of acquired drug resistance [7-9]. In addition ~10-20% of patients with EGFR mutations fail to respond to first-line EGFRi and the mechanisms underlying this intrinsic resistance are unclear [10].

In this review, we provide an overview of the synthetic lethal approaches that have revealed signalling network mechanisms which drive both oncogene and non-oncogene addiction in EGFRi-resistant NSCLC [11, 12]. We offer a perspective on the challenges faced when directing these findings towards the development of clinical therapies and offer potential solutions to overcome these issues including exploiting signalling plasticity and harnessing the principles of clonal evolution for designing novel strategies to tackle resistance.

NSCLC and EGFR inhibitor therapy

Activating EGFR mutations occur with a frequency of ~15% in lung adenocarcinoma with an enrichment in patients of East Asian descent (10% in Caucasian versus 40% in Asian populations) [13]. 90% of EGFR activating mutations are found within the kinase domain, which spans exon 18-21. The exon 21 L858R substitution and in-frame exon 19 deletions between and including residues 746-750 are the most common aberrations making up 85% of EGFR activating mutations [10]. A less frequent exon 20 insertion mutation occurs in 4% of mutant EGFR lung adenocarcinoma patients [14]. While EGFR mutations are present in multiple cancer types [15], this spectrum of kinase domain mutations appears to be exclusive to lung cancer.

First generation EGFRi gefitinib and erlotinib target the ATP binding site of the EGFR kinase by competitive reversible inhibition and are used to exploit the oncogene dependency for activating EGFR mutants in lung adenocarcinoma. These drugs are currently approved for first line treatment in patients harbouring activating EGFR mutations and have a remarkable objective response rate (ORR) of ~80% [5]. In the remaining ~20% of patients, the reasons for a lack of response are largely unclear, but among these EGFR exon 20 insertions, and BIM and PTEN deletions have been associated with resistance to EGFRi therapy [16-18]. In patients who do initially respond to EGFRi therapy, resistance invariably develops and relapse occurs within 16 months [4]. Acquisition of a secondary substitution gatekeeper mutation in EGFR (T790M) is the dominant mechanism of resistance in ~60% of treated patients [19]. In addition to T790M, other mechanisms of acquired resistance include MET and human epidermal growth factor receptor 2 (HER2) amplification, PIK3CA and BRAF mutations [20]. Activation of IGF-1R has also been observed in preclinical models of resistance to first generation inhibitors [21, 22] Histological alterations such as transformation to Small Cell Lung Cancer (SCLC) and Epithelial-Mesenchymal Transition (EMT) have additionally been reported as mechanisms of acquired EGFRi resistance [23, 24]. Likewise, the AXL receptor has been shown to contribute to acquired EGFRi resistance

in lung cancer [25]. AXL upregulation in tumour xenografts is accompanied with an EMT signature, hinting at a potential role of AXL in promoting EMT as a mechanism of resistance in lung cancer. An in-depth discussion of the distinct mechanisms of EGFRi resistance is out of the scope of this article and interested readers should read these excellent reviews for a comprehensive discussion on this topic [20, 26, 27].

Unlike the first generation drugs, second generation EGFRi, such as afatinib, bind irreversibly to EGFR via a cysteine residue (C797) in the kinase domain. While afatinib has good activity in the first line setting, it has limited clinical efficacy in the context of acquired T790M EGFR mutation with <10% ORR observed in relapsed patients [28]. The reason for this lack of efficacy is due to dose limiting toxicities caused by non-selective activity of afatinib towards wild-type EGFR and limited inhibition against the T790M gatekeeper mutant, thereby reducing its relevance as a second line treatment [28]. In response to the ineffectiveness of second generation inhibitors, third generation therapies that bind irreversibly to the C797 residue, but are capable of binding to sensitizing EGFR mutations as well as the T790M gatekeeper mutation were developed. Osimertinib (AZD9291) is currently the only third generation irreversible EGFRi approved by the FDA, with an ORR of 61% in patients harbouring a T790M mutation [6, 29]. The most common mechanism of osimertinib resistance is a single amino acid substitution of C797S, acting as a blanket resistance mechanism to block all irreversible EGFR inhibitors [8]. A fourth generation allosteric inhibitor, EAI045, to combat the C797S mutation is currently under development [30]. Additional minor resistance mechanisms to osimertinib identified include HER2 amplification and SCLC transformation, which overlap with mechanisms driving resistance to gefitinib and erlotinib [31, 32]. As seen with previous generations of EGFRi, a proportion of patients display intrinsic resistance to osimertinib, despite the presence of EGFR mutations predictive of drug response [8].

Although there have been significant advancements in lung cancer treatment with EGFRi therapy, overall survival has not improved in these patients [33]. The clinical pathway for the treatment of patients who are resistant to EGFRi therapy is limited (Fig. 1). This is due to the lack of salvage therapies to combat acquired EGFRi resistance in the non-T790M setting and also patients who harbour intrinsic resistance to this class of drugs. The quest for developing new therapies to overcome both acquired and intrinsic resistance is moving away from single mutations towards exploiting alternative signalling pathways that drive oncogene and non-oncogene addiction in EGFRi resistant cancer. With each successive generation of EGFRi, the variety, heterogeneity and complexity of these signalling networks broaden. The manner in which resistance is managed has to adapt to engage this rapidly evolving landscape where mutational analysis may no longer be sufficient to fully address the challenge of resistance in the clinic. Adaptive signalling networks which reinforce EGFR downstream effectors or promote non-oncogene addiction pathways to subvert EGFRi therapy need to be mechanistically defined for effective translation into clinical applications. A useful strategy to uncover these signalling networks in EGFRi resistance is through large-scale synthetic lethality screens.

Synthetic lethality as a means to uncover new pathway dependencies in EGFRi resistant NSCLC

The synthetic lethality strategy

Many studies which have set out to explore dependencies of EGFRi-resistant lung cancer have been based on the concept of synthetic lethality. The first description of synthetic lethality can be found as early as 1922, when Calvin Bridges observed that the presence of two mutations together in a single fruit fly was lethal, while either mutation alone had no effect [34]. This phenomenon has since been observed in individual cells in which the loss of function of two genes results in cell death, whereas independent loss of either gene alone permits the cell to remain viable [35, 36]. This forms an attractive concept in the context of cancer, where loss of function mutations in tumour suppressor genes are common, yet by

themselves are considered notoriously “undruggable” by pharmacological agents [37]. In this scenario, the theory is that a potentially dangerous functional loss which arises as a side effect of a tumour suppressor mutation is buffered by a compensatory pathway, one whose activity may otherwise have been non-essential in a normal cell. When revealed, this unique dependency of the cancer cell represents an Achilles’ heel that might be exploited by therapeutics. Indeed, this situation has been very successfully demonstrated by work elucidating the role of PARP in DNA repair. PARP activity was found to be essential only in the context of *BRCA1* or *BRCA2* deficiency, affording PARP inhibitors an effective therapeutic window in cancers harbouring these tumour suppressor mutations [38].

This specific form of genetic buffering by cancer cells, as opposed to a direct functional redundancy shared between two pathways, is sometimes referred to as “induced essentiality” [39]. The definition of synthetic lethality can be more loosely applied, however, to simply describe any interaction specific to cancer cells which is lethal when disrupted. This extension of the concept is not necessarily restricted to loss of function mutations and has been employed as a strategy to look for dependencies in cancers which are addicted to activating mutations in oncogenes. A prime example of this is the exhaustive search using both RNA interference (RNAi) and chemical libraries for vulnerabilities in cancers with activating mutations in RAS, an oncogene which has proved extremely difficult to target directly [40, 41].

In the context of mutant EGFR lung cancer, synthetic lethality can be broadly used to describe one of two types of interaction: 1) genes which are specifically required in cells with an activating EGFR mutation versus cells containing wild-type EGFR and 2) genes which, when disrupted, are synergistically lethal in combination with EGFRi treatment (Fig. 2). For the former, the identified hits hinge upon the unique requirements of EGFR-dependent cells. This could include specific downstream components of the mutant EGFR signalling network itself or, more generally, genes associated with maintaining cell survival under oncogenic

stress, for example regulators of protein synthesis, metabolism, DNA damage and replication – a feature of cancer cells described as 'non-oncogene addiction' [11]. For the second type of interaction, potential hits might be concentrated on genes which modulate sensitivity to EGFR inhibition, including pathways which share functional redundancy with EGFR signalling or distinct bypass signalling routes that facilitate escape from mutant EGFR as the primary oncogenic driver. These categories of synthetic lethal interactions are by no means mutually exclusive, and there may be genes that overlap, which perhaps have modest effects on cell growth when repressed alone but greater potency when combined with EGFRi. Nevertheless, this conceptual framework can be useful to consider when designing studies to investigate resistance to EGFRi in lung cancer. Although different study designs may be biased towards specific forms of synthetic lethal interaction, their common goal is to uncover any new vulnerability that might be exploited to overcome resistance to EGFRi therapy in NSCLC.

Constructing a library of candidate genes to probe for synthetic lethal interactions associated with EGFRi resistance can be hypothesis-driven. For example, several studies have focused on profiling the EGFR 'interactome', which is the network of proteins that interact either directly or indirectly with EGFR, as a source of potential targets [42-45]. Alternatively, phosphoproteomic profiling of mutant EGFR signalling networks in lung cancer models may provide pathway-specific candidates for inclusion into targeted screens [46, 47]. For practical reasons including speed, cost and convenience it can be beneficial to focus on a smaller subset of genes in screening approaches. An alternative to targeted EGFR-centric screens is to carry out whole kinome- or genome-wide screens. These libraries do not rely upon *a priori* knowledge of the EGFR network and because of their unbiased coverage, they have the potential to reveal completely unexpected synthetic lethal interactions or implicate genes with unassigned or poorly understood functions; minimising the possibility of missing potential hits for follow up study. Given the inherent noise in kinome- or genome-wide

screens however, caution must be taken to rigorously validate hits and eliminate false positives.

These screening approaches have been applied to compare dependencies in *in vitro* lung cancer cell line models harbouring wild-type versus mutant EGFR and extended into models of both intrinsic and acquired resistance to EGFRi. Here, we review recent studies that demonstrate the use of large-scale synthetic lethality and profiling approaches to investigate EGFRi resistance in lung cancer and highlight the main signalling pathways which have been implicated in driving resistance (Table 1).

Key pathway dependencies associated with EGFRi resistance

NF-κB

Nuclear factor-kappa B (NF-κB) is a transcription factor composed of homo- or heterodimers of different members of the NF-κB family [48]. NF-κB activity can impact multiple signalling pathways associated with apoptosis and cell survival and is frequently upregulated in tumours. Activation of the NF-κB pathway has been linked to chemotherapy resistance, highlighting its potential as a therapeutic candidate [49, 50]. Recent work suggests that NF-κB can also play a role in promoting resistance to EGFRi treatment in NSCLC.

Bivona and colleagues performed a pooled short hairpin RNA (shRNA) screen in the H1650 cell line using a library of over 2000 genes previously characterised as ‘cancer-related’ [51]. H1650 is a lung adenocarcinoma cell line that models intrinsic resistance as it harbours an exon 19 deletion EGFR mutation yet is insensitive to EGFRi treatment through mechanisms that are not completely understood [52, 53]. By performing the shRNA screen in the presence of erlotinib, the authors sought to identify genes which, when silenced, were able to promote EGFR dependency and restore EGFRi sensitivity. From this screen, 18 genes related to the NF-κB and Fas death receptor signalling pathways were found to be capable of sensitizing the cells to erlotinib. Fas, also known as CD95, is a cell surface receptor which

initiates a caspase-mediated apoptotic response and activates the NF-κB pathway when stimulated with Fas ligand [49]. However, recent studies have shown that loss of Fas expression can also illicit a cell death response in tumours [54, 55]. A subset of the NF-κB- and Fas-pathway genes were further validated in two additional models: the II-18 lung cancer cell line, which despite an L858R mutation is relatively insensitive to EGFRi treatment and an isogenic human bronchial epithelial cell (HBEC) system engineered with either an exon 19 deletion or a L858R EGFR mutation. Moreover, the authors showed that tumour specimens from a cohort of 52 mutant EGFR positive patients harbouring resistance to erlotinib but lacking the T790M mutation displayed increased NF-κB activation which correlated with a worse PFS. This study demonstrates the potential of NF-κB as a co-target with EGFR, although the efficacy of such a treatment may be dependent on the extent of NF-κB activation within individual tumours.

More recently, Sudo *et al*. also showed an involvement of the NF-κB pathway in EGFRi resistance [56]. A genome-wide shRNA synthetic lethality screen in combination with gefitinib treatment was performed in the H1975 NSCLC cell line, which contains concurrent L858R and T790M EGFR mutations. Silencing of PRKCSH, a gene which encodes the non-catalytic β-subunit of glucosidase II (GIIβ) and is involved in protein translocation at the endoplasmic reticulum, was found to have the most potent synthetic lethal interaction with gefitinib, although the mechanism underlying this dependency remains to be determined. By using a pathway interaction database to analyse the shRNA screen results, the authors also uncovered a gene signature involved in CD27 signalling which was strongly associated with gefitinib-induced cell death. CD27 is a receptor of the tumour necrosis factor (TNF) family which activates the NF-κB pathway [57]. There is also evidence to suggest that the tyrosine kinase c-Src is able to activate NF-κB via phosphorylation of IκBα [58]. Based on this rationale, the authors used a combination of gefitinib and dasatinib, a Src family tyrosine kinase inhibitor, to indirectly inhibit NF-κB activation and showed a synergistic reduction in H1975 cell growth. However, given that dasatinib is a broad spectrum inhibitor which can

block multiple tyrosine kinases, it is not possible to conclude whether NF-κB inhibition was the specific synthetic lethal event which restored gefitinib sensitivity in this particular drug combination.

Indeed, one barrier to investigating the involvement of NF-κB signalling in EGFRi resistance has been the lack of inhibitors which directly target NF-κB [59] . Often proteins which target the upstream IκB kinase (IKK) family or proteasome inhibitors are used to suppress the NF-κB pathway, but these compounds lack selectivity. To address this shortcoming, in a recent follow-up study by the Bivona group, the inhibitor PBS-1068 which directly targets the RELA (p65) subunit of NF-κB was validated as a potential candidate for tackling EGFRi resistance [60]. PBS-1068 was shown to block RELA binding to DNA and treatment with the drug was able to repress the expression of canonical NF-κB target genes. Treatment of II-18 cells with PBS-1086 enhanced the response to erlotinib and induced apoptosis *in vitro* and *in vivo*. The efficacy of this strategy was additionally assessed in a model of acquired resistance where an EGFRi sensitive cell line HCC827 was exposed to prolonged erlotinib treatment until drug resistance developed *in vitro*. Out of 12 erlotinib resistant HCC827 subclones generated, half displayed an upregulation of NF-κB activity and erlotinib sensitivity was restored by treatment with PBS-1086. These preliminary findings raise the exciting possibility of an NF-κB inhibitor and EGFRi combination as a potential strategy to combat both intrinsic and acquired EGFRi resistance in patients.

STAT3

The Signal Transducer and Activator of Transcription 3 (STAT3) transcription factor is a major driver of oncogenic signalling and drug resistance across multiple cancer types [61]. For instance, interleukin 6 (IL-6) activation of the STAT3 pathway is a known mechanism of targeted therapy resistance in oncogene-addicted cell line models driven by EGFR, HER2, Anaplastic lymphoma kinase (ALK), MET and KRAS [62]. As part of the investigation into the mechanisms through which NF-κB promotes EGFRi resistance, the previous study by the

Bivona group also observed that NF-κB activation led to the production of IL-6 and subsequent activation of the STAT3 pathway in II-18 cells upon erlotinib treatment [60]. Treatment with PBS-1068 was sufficient to block IL-6 production and prevent STAT3 phosphorylation. Thus, inhibiting the IL-6-STAT3-NF-κB signalling axis appears to be key in restoring sensitivity to EGFRi in otherwise resistant NSCLC.

STAT3 itself was identified as a synthetic lethal hit with EGFRi treatment in an earlier study by Astsaturov *et al.* [42]. The authors constructed an *in silico* network of proteins centred around EGFR by integrating multiple databases to identify proteins which have functional interactions with EGFR. Using this network, 638 genes were prioritised and a targeted short interfering RNA (siRNA) screen was performed in the A431 cervical adenocarcinoma cell line, which has high wild-type EGFR expression levels and is consequently addicted to EGFR signalling for survival. Taking this hypothesis-driven approach, the study found a densely connected core network of EGFR-related genes which were able to modulate sensitivity to erlotinib and cetuximab, an EGFR targeting antibody. Among these hits, knockdown of STAT3 was identified as one of the most sensitizing to EGFRi. Treatment with Stattic, a small molecule inhibitor of STAT3, synergised with erlotinib to inhibit A431 cell growth. Unfortunately, there is currently a lack of direct STAT3 inhibitors in clinical trials due in part to the challenges of designing inhibitors that disrupt protein-protein interactions driving STAT3 dimerization, making this strategy difficult to implement in the clinical setting.

Wnt

The Wnt family of ligands impact a diverse range of cellular process and dysregulation of the Wnt/β-catenin pathways are known to occur in many cancer types such as colorectal cancers [63]. A genome-wide shRNA synthetic lethal screen performed by Casás-Selves *et al.* showed that several positive regulators of the canonical Wnt signalling pathway promoted cell survival upon EGFR inhibition [64]. The shRNA dropout screen was performed in combination with gefitinib treatment in two lung cancer cell line models, H322C which

expresses wild-type EGFR and displays intermediate sensitivity to gefitinib, and HCC4006 which harbours an exon 19 deletion EGFR mutation and is highly sensitive to gefitinib treatment. Based on this experimental design, the authors sought to identify pathways able to maintain a residual surviving population of cells undergoing EGFRi treatment. After next generation sequencing and bioinformatics analysis, the study found multiple shRNA hits linked to the Wnt/Tankyrase/β-catenin signalling pathway, including the genes encoding the poly-ADP-ribosylation enzymes tankyrase 1 (TNKS1) and tankyrase 2 (TNKS2). Mice bearing tumour xenografts with shRNAs targeting TNKS1 showed significantly more growth inhibition upon gefitinib treatment compared to control. A pharmacological inhibitor of tankyrase activity, XAV939, was similarly capable of synergistically inhibiting cell growth in combination with gefitinib treatment in HCC4006 cells and this effect could be partially rescued by overexpression of a mutant activated form of β-catenin.

In a recent follow up study, gefitinib treatment combined with a novel dual inhibitor of TNKS1 and TNKS2, AZ1366, was also shown to synergistically inhibit NSCLC growth using both *in vitro* and *in vivo* models [65]. Although the degree of synergism between EGFR and tankyrase inhibition was variable across different NSCLC cell lines, it correlated with the extent of Wnt signalling pathway modulation, implicating a direct involvement of this pathway in promoting cell survival in the presence of EGFRi. However, the mechanistic basis of the synthetic lethal interaction between the EGFR and Wnt signalling pathways are unknown. Future work should focus on characterising the molecular mechanisms responsible for the synergy between these pathways and establishing biomarkers to identify the Wnt signalling-dependent tumours which are most likely to benefit from combined tankyrase and EGFR inhibition.

Src family kinases (SFKs)

Src belongs to a group of related non-receptor tyrosine kinases known as the Src family kinases (SFKs) which influence a wide array of fundamental processes including cell

proliferation, differentiation and migration; and as a result aberrant SFK signalling is capable of contributing to different features of cancer development [66]. SFKs were highlighted as potential EGFR co-drivers in NSCLC in a phosphoproteomic profiling study performed by Yoshida *et al.* [46]. By comparing the phosphoproteomic profiles between sensitive and resistant cell lines, this study sought to determine the pathways cooperating to promote resistance to EGFRi. Mass spectrometry was used to evaluate the tyrosine phosphoproteome of PC9 cells possessing a sensitizing exon 19 deletion EGFR mutation versus a gefitinib resistant counterpart which had acquired a T790M mutation following long-term exposure to gefitinib. Though the levels of phosphorylation on a large number of proteins were distinct between resistant and sensitive cell lines and were also perturbed by EGFRi treatment, tyrosine phosphorylation in a smaller group of 31 proteins, including the SFK proteins, remained relatively stable after the acquisition of resistance, whilst also being unaffected by EGFRi treatment. The authors hypothesised that this persistent SFK signalling might operate in parallel to EGFR signalling to maintain cell survival. A combination of afatinib and the SFK inhibitor dasatinib was shown to be effective in NSCLC cells with the T790M mutation, demonstrating that cooperation of both pathways drives cell growth in the acquired resistance setting. Based on these data, a phase I clinical trial is ongoing to determine the efficacy of the afatinib and dasatinib combination in patients with first-generation EGFRi acquired resistance (NCT01999985).

A focused shRNA kinome screen targeting ~600 kinases carried out in the H1650 cell line also found that Src inhibitors can be effectively used in combination with EGFRi [67]. Seven kinases were shown to be essential survival genes in this cell line model: EGFR, CDK6, MARK3, PBK, TBK1, DDR1 and EPHA4. There is no obvious link between the biological functions of these kinases and whether their synthetic lethal interaction with mutant EGFR occurs through common or distinct mechanisms is unknown. In an attempt to define pharmacological compounds which might connect these seven kinases, the authors applied an algorithm known as the Kinase Connectivity Map (K-Map) which uses a reference

database of gene expression profiles to rank potential pharmacological agents based on a list of queried targets [68]. Intriguingly, K-Map analysis identified bosutinib, a dual Abl and Src inhibitor, as a highly ranked candidate inhibitor linking these kinases. While combined bosutinib and gefitinib treatment was effective at inhibiting the proliferation of H1650 and H1975 cell lines, the exact mechanism of action of this combination has yet to be defined, highlighting our lack of understanding of the precise details of how many of these synthetic lethal interactions with EGFRi operate.

SWI/SNF complex

The SWI/SNF chromatin remodelling complex is composed of multiple protein subunits and functions to organise nucleosomes in an ATP-dependent manner, facilitating many DNA regulatory processes, including gene expression and DNA replication and repair [69]. Pan-cancer next-generation sequencing analyses show that ~20% of all human cancers harbour deleterious mutations in SWI/SNF protein subunits, suggesting a possible functional role as tumour suppressors [70, 71]. Emerging evidence demonstrates that the SWI/SNF complex can influence tyrosine kinase signalling and modulate responses to targeted therapies [72-74]. In lung cancer, this has recently been shown by the observation that loss of SMARCE1, a SWI/SNF subunit, can enhance EGFR expression levels and confer resistance to MET and ALK inhibitors in NSCLC cell lines [72].

A recent functional genomics screen by Liao *et al.* has also implicated SWI/SNF as a modulator of EGFR dependency in NSCLC cells [75]. The authors constructed an *in silico* library by using an algorithm called TUSON (Tumour Suppressor and Oncogene) to identify potential tumour suppressors or oncogenes in EGFR mutant lung cancer cell lines [76]. CRISPR and shRNA were used to target ~500 candidate tumour suppressor genes derived from the *in silico* library in PC9 cells treated with gefitinib for 17 days. By using the two orthogonal techniques of CRISPR and RNAi, the authors aimed to increase the breadth of genes which might be captured by using only one approach. For example, genes which

require a complete depletion before a phenotype can be observed would be missed by shRNA, whilst genes which are essential for cell survival would not be identified by CRISPR. Among the top 30 scoring genes in both CRISPR and shRNA screens were three SWI/SNF complex subunits: PBRM1, ARID2 and ARID1A, whilst two additional subunits, ARID1B and SMARCB1, were identified from the CRISPR screen only. The authors went on to demonstrate that loss of PBRM1 was able to sustain AKT survival signalling in PC9 cells despite the presence of gefitinib, ultimately conferring drug resistance. However the mechanism by which PBRM1 regulates AKT signalling is not known. Moving forward, in order to translate these observations into clinical applications, it will be crucial to gain further insights into the interplay between individual SWI/SNF subunits and the EGFR network so as to better characterise their effects on cell signalling and EGFRi resistance. Nonetheless, this study highlights the importance of integrating orthogonal techniques in large-scale screening studies to achieve a broader view of pathways which can modulate response to EGFRi therapy.

Canonical EGFR signalling components

When searching for dependencies specific to EGFR addicted lung cancers, aside from EGFR itself, closely interacting proteins which are involved in propagating EGFR signalling could represent an additional source of candidate targets. This hypothesis was explored in a study by Li *et al.*, which took an integrated proteomic profiling and functional screening approach to identify synthetic lethal interactors specific to mutant EGFR [43]. An EGFR interactome was constructed using a comprehensive series of tandem affinity purification-liquid chromatography-mass spectrometry (TAP-LC-MS/MS) experiments, in which the exon 19 deletion E746-A750 mutant EGFR was used as a bait protein to pull down protein interactors in two model cell lines which endogenously express this EGFR mutation, PC9 and HCC827. Based on the initial pull down data, proteins which closely interacted with mutant EGFR were then deployed as baits for a second round of TAP-LC-MS/MS experiments designed to increase network depth, ultimately resulting in a detailed EGFR

interactome of 263 proteins. An siRNA library to target a subset of these proteins was deployed to assess the essentiality of EGFR interactome members to the survival of a panel of wild-type and mutant EGFR lung cancer cell lines. A core network of 14 proteins were commonly identified as essential genes across all cell lines tested and of these, 9 targets affected cell viability to a significantly greater extent in mutant versus wild-type EGFR lung cancer cell lines. Among these positive hits, knockdown of EGFR itself, and also number of downstream signalling components related to the mitogen-activated protein kinase (MAPK) pathway including (Growth factor receptor-bound protein 2 (GRB2), SHC-transforming protein 1 (SHC1) and mitogen-activated protein kinase 12 (MK12)) had the most potent effect on cell viability.

Expanding further, the authors hypothesised that if models of acquired EGFRi resistance driven by secondary mutations maintain a dependence on EGFR signalling for growth, these core components of the EGFR network should similarly remain essential for survival. To test this idea, they showed that knockdown of the EGFR signalling adaptors GRB2 and SHC1 was essential in laboratory-derived gefitinib-resistant PC9 cells harbouring a T790M mutation. In contrast, in similar experiments performed in the gefitinib-resistant HCC827 model where MET amplification is the primary mechanism of resistance [77], the effect of silencing of EGFR interactors on cell viability was reduced. This data demonstrates that dependence on canonical EGFR signalling becomes less central in resistant mechanisms that are distinct from secondary EGFR mutations, such as MET activation.

Various EGFR signalling components, including SHC1, have similarly been identified in previously discussed large-scale synthetic lethal studies [42, 45], supporting the idea that proteins proximal to EGFR might act as alternative targets to overcome EGFRi resistance. However, the application of these findings in a clinical setting may be limited by the lack of pharmacological agents capable of acting on adaptor proteins which do not possess intrinsic enzymatic activity. In addition, targeting the proteins proximal to EGFR signalling is likely to

be restricted to situations in which a largely EGFR-driven phenotype is maintained after development of resistance.

Outstanding challenges and emerging solutions

Despite a comprehensive knowledge base of synthetic lethal pathways associated with drug resistance, the only drug approved for patients who progress on EGFRi therapy is osimertinib. There continues to be a lack of salvage therapy options available for patients who become resistant to first generation inhibitors but do not harbour the T790M gatekeeper mutation. Several outstanding challenges remain which limit our ability as a field to translate the large body of synthetic lethality data described above into effective therapies for patient benefit. In this section we describe some of these key challenges and define emerging network biology solutions that hold the potential to progress these discoveries beyond the confines of the laboratory.

Unravelling network complexity and context dependency

As discussed in the previous section, exploiting EGFR interactome and phosphoproteomic data in combination with synthetic lethality screens has to some extent been successful in identifying key genes which act in concert or in parallel with EGFR signalling to confer drug resistance (Fig. 3). Why is it then that translating these synthetic lethal interactions into effective clinical strategies still remains elusive? Part of the problem is the significant gap in our knowledge of how individual synthetic lethality genes converge on core cell signalling networks. For instance, for many of the synthetic lethal genes discussed, their connection to the EGFR network is unknown. Moreover, there is poor overlap of synthetic lethal genes identified across different large-scale studies which may be due to differences in experimental set up including RNAi strategy, phenotypic assays and cell line models used, amongst others. It is also necessary to determine the biological context in which these synthetic lethal interactions occur and use this information to stratify lung cancer patients

who might benefit the most from synthetic lethality-based therapy. An emerging approach which may prove useful in both determining the impact of synthetic lethal genes on cellular signalling networks and resolving the observed differences between synthetic lethal studies is the use of computational tools to integrate multiple datasets, predict lethal interactions within specific genomic/epigenomic contexts and map these interactions onto existing databases of cell signalling networks.

A good example of such a computational approach is the recently developed Data-mining synthetic lethality identification pipeline (DAISY) [78]. DAISY was designed using three basic principles to statistically infer synthetic lethal interactions at a genome-wide level. First, genomic data including somatic copy number alterations and mutations were used to identify pairs of genes which are co-inactivated at a much lower frequency than by chance. This relies on the assumption that the concurrent loss of these genes is unfavourable and therefore rarely seen as a result of selective pressure. Second, data from shRNA essentiality screens and accompanying transcriptomic profiles were used to identify cases where underexpression of one gene resulted in an induced essentiality of a second gene. Lastly, given that synthetic lethal gene pairs are often linked by closely related biological processes, it was anticipated that they should be frequently co-expressed. Using DAISY, the authors predicted and validated novel synthetic lethal interactions with the well characterised Von Hippel-Lindau (VHL) tumour suppressor. Importantly, a combinatorial analysis was used to construct a genome-wide network of ~3000 different synthetic lethal interactions, highlighting biological pathways with the highest densities of interactions. This global synthetic lethal network was capable of predicting the response of cancer cell line models to RNAi or drugs based on their genomic profiles. In addition, the network was used to predict the outcome of breast cancer patients based on the assumption that the under-expression of two genes whose concurrent loss is expected to be synthetically lethal would be disadvantageous to tumour growth. Indeed, the authors were able to show a correlation between an overall

increased frequency of synthetic lethal gene pair loss within tumours and better prognosis in breast cancer.

Another resource, Synthetic Lethal Database (SynLethDB), has been developed in an attempt to collate our current knowledge of synthetic lethal interactions across multiple disparate data sources [79]. Similar to DAISY, SynLethDB also integrates genomic, transcriptomic and drug sensitivity data from cancer cell lines to guide the prediction of drug efficacy given specific synthetic interactions within particular genomic contexts. A web interface provides additional utility to explore synthetic lethal interactions at the biological network level. This includes the ability to perform gene set enrichment analysis (GSEA) on the subset of genes known to possess synthetic lethal relationships with a particular query, meaning broader mechanistic insight can be quickly gained following the identification of individual genes of interest from traditional screening methods.

Applying these tools to data from synthetic lethal screens of EGFRi resistant cells will help to visualise the relationship of seemingly distant genes with canonical EGFR signalling pathways. Ultimately, this will prove vital in reconciling distinct gene hits from different studies onto robust sets of shared synthetic lethal pathways important for maintaining survival during EGFR inhibition. These strategies could overcome some of the issues associated with non-overlapping gene targets or context-dependent interactions that currently limit the utility of synthetic lethality data in the pre-clinical and clinical settings [41]. It is important to note that synthetic lethal interaction prediction and mapping by tools such as DAISY and SynLethDB is still at its infancy and will only improve as the amount of input data from cell line and patient studies increases over time. Coupled with the growing availability of comprehensive genetic and epigenetic information, these resources could be invaluable in identifying predictive biomarkers to stratify patients who are likely to benefit from therapies based on synthetic lethality principles.

Exploiting signalling plasticity

A hallmark of intrinsic and acquired EGFRi resistance is signalling plasticity, which is defined as the innate capacity of signalling networks to adapt in response to perturbations, including drug challenge or gene silencing, as a means of maintaining robust tumour cell survival [80]. This includes both short-term rewiring such as kinome reprogramming within hours of drug administration as well as long-term adaptive responses in drug persister cells during the acquisition of kinase inhibitor resistance [81-84]. Signalling plasticity is often considered a challenging obstacle in the development of therapies to overcome resistance; and as illustrated by examples provided in the previous sections, an enormous effort has gone into characterising these adaptive responses in order to identify resistance-causing bypass pathways. In contrast, several recent studies have highlighted signalling plasticity as an overlooked opportunity in devising new strategies to tackle EGFRi resistance [85, 86]. These studies are predicated on the idea that drugs can rapidly rewire networks, enabling tumour cells to achieve a new "signalling state" which induces novel vulnerabilities and synthetic lethal interactions that can be exploited for cancer therapy.

In one such study, Lee *et al*. systematically assessed the phenotypic effects of combinations of seven genotoxic drugs and eight signal transduction inhibitors in cancer cells [85]. Unlike other efforts which focus on simultaneous testing of pair-wise combinations [87, 88], the authors in this study also varied the dosing schedule, the order of drug presentation and dose duration. They made the interesting observation that pre-treating triple negative breast cancer (TNBC) cells with erlotinib followed by administration of the DNA damaging agent doxorubicin induced a 5-fold increase in apoptosis compared to treatment of either drug alone or both drugs in combination. Intriguingly, pre-treating cells with doxorubicin followed by erlotinib did not provoke a similar apoptotic increase. This led the authors to posit that EGFR pathways may be suppressing the signalling networks governing susceptibility to DNA damaging agents. To test this hypothesis, Lee and colleagues undertook a large-scale collection of temporal measurements comprising 35 signalling nodes that lie in pathways

important for cellular responses to genotoxic stress in breast cancer cells subjected to erlotinib and doxorubicin treatment. This signalling data was integrated with phenotypic measurements using computational approaches and revealed that the pro-apoptotic effector caspase-8 was key to the mechanism by which erlotinib sensitized TNBC cells to doxorubicin. In this model the authors propose that under baseline conditions, EGFR signalling suppresses caspase-8 activation and that inhibition of EGFR with erlotinib results in the reactivation of apoptotic pathways which render tumours cells more susceptible to DNA damage. Importantly, this finding was not isolated to TNBC cells but was also active in mutant EGFR NSCLC cells [85], suggesting that this sequential treatment approach may have utility in overcoming EGFRi resistance in lung cancer (Fig. 4A).

The concept of exploiting signalling plasticity has also been deployed in the context of immunoregulatory modulation in NSCLC. Immune checkpoint inhibitors such as the programmed cell death protein 1 (PD-1) blocking antibodies nivolumab and pembrolizumab have provided significant overall survival benefit in ~20% of NSCLC patients and are approved for second-line therapy in patients who have progressed on platinum chemotherapy [89, 90]. Most recently, based on data from the KEYNOTE-024 trial, pembrolizumab has been also approved for first-line therapy in programmed death ligand-(PD-L1)-positive NSCLC patients [91]. Preclinical and clinical studies have found several biological factors to be associated with immune checkpoint inhibitor response, including PD-L1 expression levels, the presence of tumour infiltrating lymphocytes (TILs), tumour mutational load and neoantigen repertoire [91-94]. The relationship between mutant EGFR status and PD-L1 expression has been controversial. Two reports demonstrate that oncogenic signalling by mutant EGFR promotes evasion of immune surveillance by upregulating PD-L1 expression and immunosuppressive cytokines in cell lines and genetically engineered mouse models (GEMMs) [95, 96]. Administering anti-PD-1 antibodies in mutant EGFR-driven GEMMs facilitates the recruitment of TILs and reduces tumour burden [96]. However, a recent retrospective analysis showed that mutant EGFR NSCLC

patient specimens harbour low levels of concomitant PD-L1 expression and CD8+ effector T cells in both the treatment naïve and acquired EGFRi resistance settings [97]. Importantly, this study found that patients with mutant EGFR-positive tumours had a shorter PFS compared to a cohort of wild-type EGFR patients when treated with PD-1/PD-L1 inhibitors, leading the authors to speculate that the lack of efficacy may be the result of a lack of pre-existing TILs within the tumour.

Building on these data, Dominguez *et al*. sought to establish if EGFR inhibition is capable of re-wiring cellular signalling to modulate immune-mediated cytotoxicity in NSCLC lines [86]. They found that short-term treatment of mutant EGFR cells with erlotinib promoted susceptibility to tumour cell killing by natural killer (NK) cells and cytotoxic T lymphocytes, which were not observed with longer-term administration of the drug. Similar to the study by Lee *et al*., the authors found that this enhanced response to immune cell attack was the consequence of the upregulation of caspase-mediated apoptosis pathways in the presence of short-term erlotinib treatment. EMT is a known mechanism of EGFRi resistance and NSCLC lines subjected to long-term treatment with EGFRi gain mesenchymal features [44, 98]. In contrast, the authors showed that the increased immune cell-mediated cytotoxicity observed with short-term erlotinib treatment was associated with a rapid and dynamic acquisition of a “more epithelial” phenotype. While causation between the establishment of a mesenchymal state and resistance to immune-mediated cytotoxicity was not definitively established, this study raises the tempting prospect that sequential administration of short-term erlotinib therapy followed by immunotherapies could delay the acquisition of drug resistance associated with long-term EGFR inhibition and promote the efficacy of immune checkpoint inhibitors in mutant EGFR NSCLC patients.

While these studies have conceptually identified new strategies in utilising EGFR inhibitors and other therapeutic agents in a sequential fashion to exploit signalling plasticity and induced synthetic lethality, we are only starting to scratch the surface of this fascinating

approach and there remains much work to be done to fully implement this concept into clinical reality. Further fundamental work is required to characterise the acute and chronic dynamic rewiring in response to EGFRi therapy, a phenomenon which has yet to be considered in synthetic lethality screening studies to date. While the studies by Lee *et al*. and Dominguez *et al*. have identified components of the apoptotic machinery as key to time-staggered therapy, the detailed mechanisms by which EGFR signalling supresses apoptotic processes remains to be defined. These studies also highlight our inadequate knowledge of the effects of drug scheduling and drug-drug interactions on signal transduction and cellular decision processes. Computational modelling of optimal drug scheduling of targeted therapeutics used in combination with cytotoxics has largely been confined to the prediction of phenotypic outputs such as tumour growth without accounting for alterations in tumour cell signalling [99-101]. To maximise the full potential of this approach, moving forward it will be necessary to integrate these pharmacokinetic and pharmacodynamics models with computational strategies that have been developed to study signal transduction networks [102]. Given that drug combinations have and will continue to be the mainstay of effective cancer therapy, future developments to optimise drug scheduling and dosing will be necessary to delay the acquisition of drug resistance while minimising treatment-induced toxicities in patients.

Harnessing the potential of tumour heterogeneity and cancer evolution

There is an increasing appreciation that cancer is a heterogeneous disease and evolutionary principles are at play during the selection of resistant tumour cell clones when patients are subjected to therapy [103]. Sensitizing EGFR mutations are considered early truncal events which represent good therapeutic targets as these mutations are theoretically present in all cells within the tumour [104, 105]. Under selective pressure, for instance in the presence of an EGFRi, secondary somatic mutations (branch events) may abolish tumour cell dependencies on truncal events, the most prominent example being pre-existing T790M mutation-containing subclones in treatment-naïve NSCLC patients [81, 82, 106]. It is now

clear that an effective cancer therapy is not just one that possesses potent tumour cell killing properties but also one that is capable of avoiding or delaying the development of drug resistance, achieving durable clinical responses. Resistant clones can emerge during prolonged exposure to TKI selection, each with distinct mechanisms of resistance; and it should be noted that many of the synthetic lethality NSCLC studies to date have only focused on population-level resistance mechanisms and short-term drug responses. The importance of tumour heterogeneity and cancer evolution in the development of drug resistance has been described in detail elsewhere and the reader is directed to recent reviews on this topic [106-108]. For the purposes of this discussion, we will focus on emerging strategies to harness cancer evolution as a means of overcoming drug resistance, which involve the utilisation of computational modelling to predict clonal trajectories, delay acquired resistance and identify newly acquired tumour vulnerabilities.

There has been significant interest in utilising computational techniques to design therapeutic strategies capable of predicting clonal trajectories and delaying the acquisition of drug resistance in NSCLC. Much of the earlier work in this area focused on varying the dose and scheduling of single agents to exploit the differential growth kinetics and fitness defects of drug resistant versus sensitive cells [109-112]. In one example, Chmielecki *et al*. utilised evolutionary mathematical approaches based on birth-death process models to assess if alternative dose scheduling regimens of EGFRi could minimise the development of acquired drug resistance in NSCLC [111]. Using PC9 cells that have acquired T790M-mediated resistance as a result of dose escalation with the second generation EGFRi afatinib; they found that in the absence of drug, resistant cells grew more slowly compared to parental cells. Exploiting this fitness disadvantage in afatinib resistant PC9 cells, the mathematical model predicted that administering low dose EGFRi in combination with intermittent high dose pulses of EGFRi would delay the development of a pre-dominant T790M containing subpopulation. They validated this prediction experimentally using high dose weekly afatinib in combination with continuous low dose erlotinib in *in vitro* experiments. This dosing

regimen doubled the time to acquisition of drug resistance compared to continuous dosing alone and prevented the growth of T790M-containing cells. While intermittent dosing appears to be effective in delaying resistance, it should be noted that T790M-independent drug resistance eventually developed with this dosing schedule [111], suggesting that additional strategies are required for salvage therapy.

Another active area of research is the use of computational models to determine optimal combination therapies to control evolutionary trajectories and modulate intratumoural heterogeneity [113-115]. While none of these approaches have thus far been applied to NSCLC, the principles identified have general implications on the design of combinatorial approaches and synthetic lethality strategies to overcome resistance derived from the clonal selection of drug resistant subpopulations within a heterogeneous tumour. The current standard of care for most TKI therapies is to administer therapy that eliminates the dominant subpopulation present at relapse. In the case of EGFRi in NSCLC, treatment with the third generation inhibitor osimertinib follows first generation erlotinib/gefitinib therapy if the re-biopsy at progression reveals the presence of a T790M mutation (Fig. 1). Recent integrated computational and experimental analyses suggest that instead of focusing on the predominant subpopulation, an alternative and perhaps more effective approach is to identify drug combinations and synthetic lethal interactions that minimise the growth of all subpopulations within a heterogeneous tumour, maintaining the naïve pre-treatment state [114, 115]. In two related studies, Zhao *et al*. employed immortalised cells from the Eμ-Myc-p19Arf$^{-/-}$ lymphoma model where mixtures of different shRNA knockdown lines were used to model a genetically heterogeneous tumour population [114]. This panel of shRNA knockdown cells have undergone comprehensive single agent dose response analysis to a range of chemo- and targeted- therapeutics [116]. Using this experimental data, the authors performed computational analysis which led them to the interesting discovery that drug combinations which are effective in minimising tumour heterogeneity may not contain single agents that are most beneficial at killing the predominant subpopulation [114, 115]. Rather

than preventing the outgrowth of specific subpopulations, they posit that it may be more desirable to identify drug combinations that are best at killing the broadest range of subpopulations (Fig. 4B) [115]. An additional important conceptual advance from these studies is that in order to accurately predict the best combinations to employ in a heterogeneous tumour, solely relying on the genotype of the predominant subpopulation is insufficient and some minimal information about the nature of the genetic makeup and heterogeneity of the tumour at diagnosis or re-biopsy is required.

The same group subsequently investigated resistance-causing mutations that accumulate in response to TKI selection and how the acquisition of such mutations over the course of tumour evolution may lead to newly acquired vulnerabilities to other drugs [117]. This is a form of synthetic lethality known as collateral sensitivity which has been previously been exploited to combat drug resistance in bacteria [118-120]. Utilising immortalised cells from a murine model of Philadelphia chromosome positive (Ph+) adult lymphoblastic leukemia (ALL) which is driven by the BCR-ABL1 fusion oncogene, they carried out a series of experiments in which the cells were subjected to dose escalating regimen with BCR-ABL1 TKIs dasatinib or bosutinib. Prior to each round of dose escalation, the cells were put through a targeted drug screen to identify agents which are capable of sensitizing the cells en route to acquiring full drug resistance. They found that in intermediate steps towards full resistance, the cells were sensitive to a number of non-classical BCR-ABL1 TKIs including crizotinib, foretinib, vandetanib and cabozantinib. Continued selection towards full resistance was accompanied by a reduction of this observed collateral sensitivity, indicating that this effect was transient in nature and not persistent. They showed that this sensitization was accompanied by the presence of a BCR-ABL1 V299L mutation and a battery of assays were used to demonstrate that this mutant was selectively inhibited by the non-classical BCR-ABL1 inhibitors over the wild-type oncogene. Unlike the examples given in the previous section, this new vulnerability was not the result of cellular rewiring and signalling plasticity but via the selection of pre-existing V299L containing subclones. It is important to note that

these vulnerabilities would have been missed by studies which focus solely on short-term drug responses or already well-established models of resistance, highlighting a shortfall in the design of many synthetic lethal screening studies to date. One of the key messages from this study is that existing kinase inhibitors which exhibit poor efficacy in wild-type kinases may display unanticipated selectivity for drug-resistant mutants. In some respects the development of osimertinib, which arose from a screen of insulin-like growth factor receptor 1 (IGF-1R) inhibitors is another prominent example of how mutant selective inhibitors can be derived from existing small molecule inhibitor panels [121].

Collectively, these studies highlight the importance of considering intratumoural heterogeneity in developing targeted therapeutic strategies to overcome drug resistance. Specifically, knowledge of the subpopulation composition, not just the predominant clone may be necessary to identify drug combinations and synthetic lethal interactions to minimise tumour heterogeneity. It is anticipated that ongoing prospective studies to map the spatial and temporal alterations in NSCLC tumour heterogeneity such as the Lung TRACERx study will provide the information required to model and ultimately predict optimal drug combinations and dosing schedules to achieve durable responses in patients [122].

Concluding perspectives

With the lack of reproducible hits between different studies and the failure to translate any of the identified targets into the clinic, synthetic lethality as a concept appears to have fallen short of its promise to identify new clinical strategies for tackling cancer and drug resistance [41]. It is vital that tangible steps are taken to address these perceived failures. Our view is that for the majority of identified synthetic lethal interactions that have been described in this review, the fundamental mechanisms by which these genes cause lethality are largely unknown. To fully harness the potential of synthetic lethality, we argue that there is an urgent need to determine the impact of these genes on both oncogene and non-oncogene signalling networks. In addition, unlike the BRCA-PARP interaction, many of the identified

synthetic lethal genes are context-dependent which are known as ‘soft’ interactions [123, 124]. Establishing the molecular basis of these context dependencies will enable one to identify additional genetic perturbations that are capable of converting these ‘soft’ interactions into more robust ‘hard’ synthetic lethality effects. Furthermore, understanding this context dependency may also provide useful information for biomarker discovery to stratify patients who are most likely to benefit from therapies based on synthetic lethal targets.

There is also a significant gap in our knowledge of how synthetic lethality fuels tumour heterogeneity and evolution. Given that it is now established that EGFRi resistance is driven by multiple resistance mechanisms within a heterogeneous tumour [81, 82, 125], it is conceivable that distinct subpopulations may require different synthetic lethal strategies in order to minimise tumour heterogeneity and prevent the outgrowth of pre-dominant sub-populations. Isolating and characterising both pre-existing and emergent synthetic lethal interactions in distinct subpopulations within a heterogeneous tumour as well as accounting for immune and stromal cells in the context of co-culture experimental systems will be necessary to effectively overcome tumour evolution associated with drug resistance. Finally, integrating synthetic lethal targets as a means of combination therapy has yet to be rigorously explored. Conceptually, the idea of combining oncogene- and non-oncogene-dependent hits is an attractive means of delaying the acquisition of drug resistance either by targeting two different lethality mechanisms in the same tumour subpopulation or by killing different subpopulations within a single heterogeneous tumour (Fig. 4C). We anticipate that tackling these challenges and exploiting phenomena such as signalling plasticity and tumour evolution represent important future steps in translating these synthetic lethality discoveries into the clinic.

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Figure Legends

Figure 1. Sequence of EGFRi treatment in patients with sensitizing EGFR mutations. Upon identification of sensitizing EGFR mutation, patients are treated with first (or second) generation inhibitors. At re-biopsy, ~60% of cases develop T790M resistance which is subsequently treated with third generation inhibitors. Emerging data indicates that a major mechanism of acquired resistance to third generation inhibitors is the acquisition of the C797S mutation and fourth generation allosteric inhibitors are currently in development to tackle this receptor variant. Additionally, the reversion to T790M back to the wildtype form of the mutant receptor also occurs, presenting an opportunity to re-challenge with first

generation inhibitors. At each stage of this sequence, intrinsic resistance is observed in some patients while acquired resistance by T790M-independent mechanisms are also depicted. For the majority of these patients, no effective salvage therapies are currently available. The points in the sequence where synthetic lethality-based targets could be exploited as salvage therapies are highlighted in red.

Figure 2. The two major forms of synthetic lethal interactions in the context of mutant EGFR and EGFRi-resistant NSCLC. (A) Lung cancer cells which harbour mutant EGFR may be uniquely dependent on some genes versus wild-type EGFR expressing cells in order to cope with aberrant EGFR signalling. (B) Lung cancer cells which harbour mutant EGFR but are insensitive to EGFRi may be dependent on additional genes in order to sustain survival in the presence of EGFRi.

Figure 3. Synthetic lethality pathways associated with EGFRi resistance. Aberrant EGFR signalling in NSCLC harbouring L858R or exon 19 deletion EGFR mutations can promote tumour growth via PI3K/Akt, RAS-RAF-MAPK and JAK/STAT pathways among others. Adaptors such as SHC or GRB2 and Src family kinases which support EGFR signalling can represent important co-targets specific to mutant EGFR cells. Other RTKs including MET and AXL mediate EGFRi resistance through aberrant activation or crosstalk to sustain oncogenic signalling. Upregulation of NF-κB can promote cell survival, mediated at least in part through production of the cytokine IL-6 and activation of JAK/STAT signalling. Activation of tankyrase via WNT signalling results in AXIN2 degradation and destabilisation of the destruction complex which targets β-catenin, permitting further oncogenic signalling. Loss of members of the SWI/SNF complex can sustain AKT signalling despite EGFRi therapy through unknown mechanisms. Although each of these synthetic lethal interactions may represent a therapeutic target, we are limited by poor mechanistic understanding of the convergence of these targets on cell signalling.

Figure 4 Strategies for combating tumour heterogeneity and acquired resistance in EGFR mutant lung cancer. (A) Exploiting signalling plasticity by pre-treating with erlotinib which induces sensitivity to DNA damaging agent doxorubicin. (B) Using computational strategies to predict combinations that broadly target the growth of all subpopulations, both pre-existing and emergent resistant cells within a tumour, rather than just the predominant resistant subpopulation. This strategy facilitates the maintenance of the pre-treatment state. (C) Combined inhibition of synthetic lethal targets with distinct mechanisms of action to eradicate both pre-existing and emergent subpopulations within a heterogeneous tumour.

Figure 1

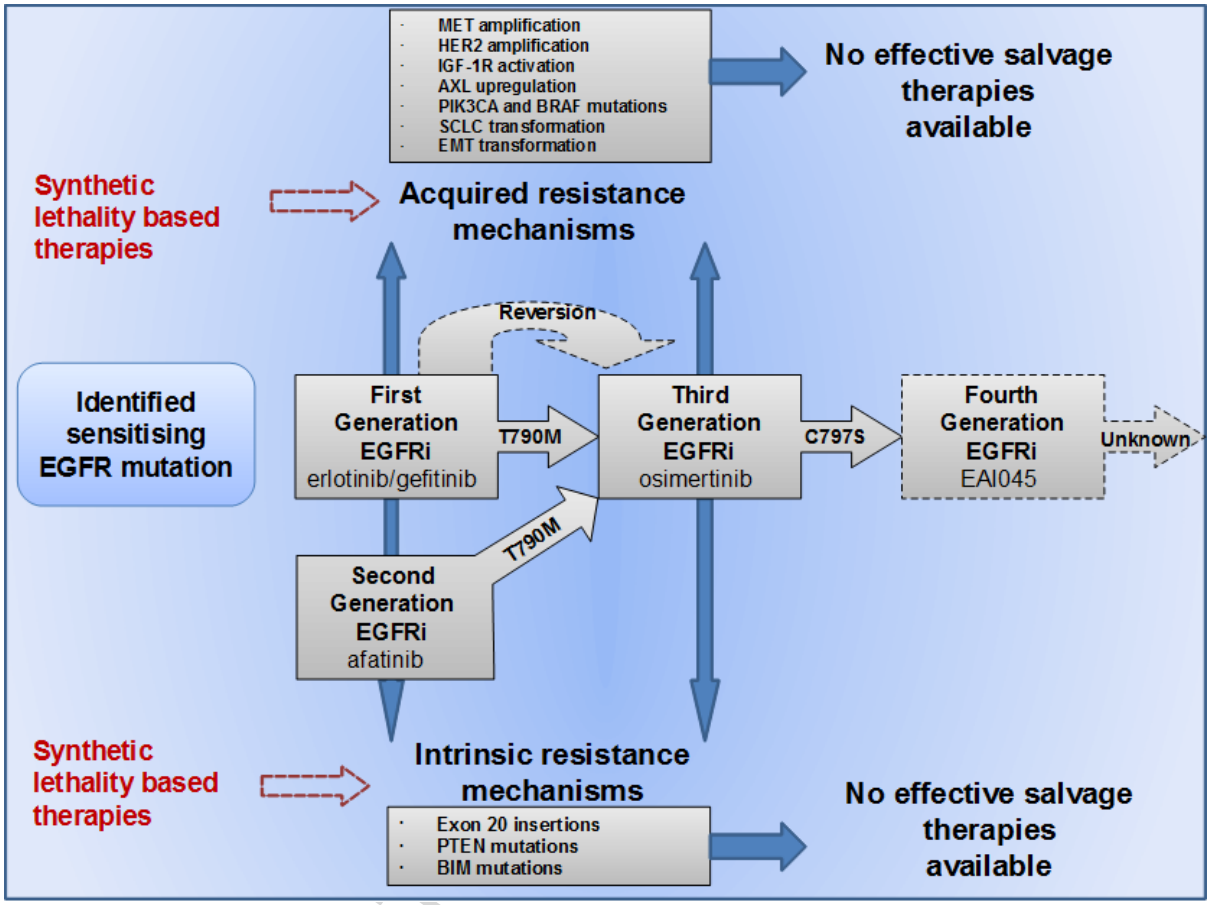


Figure 2

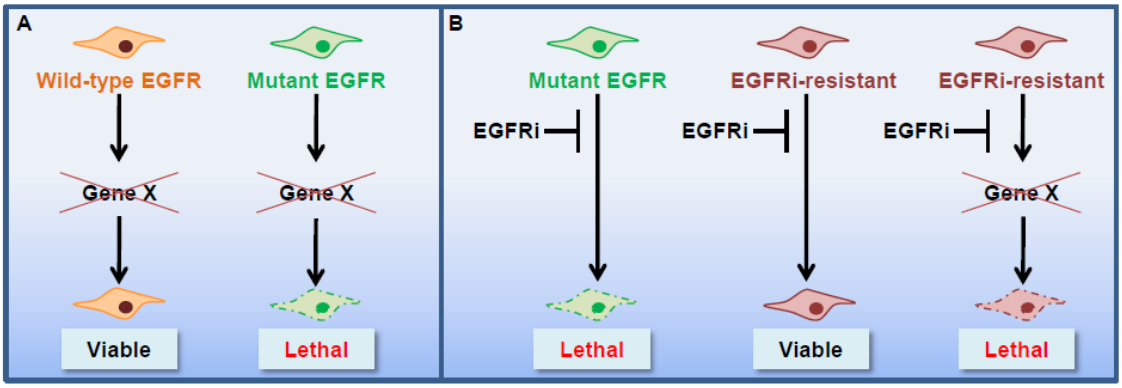


Figure 3

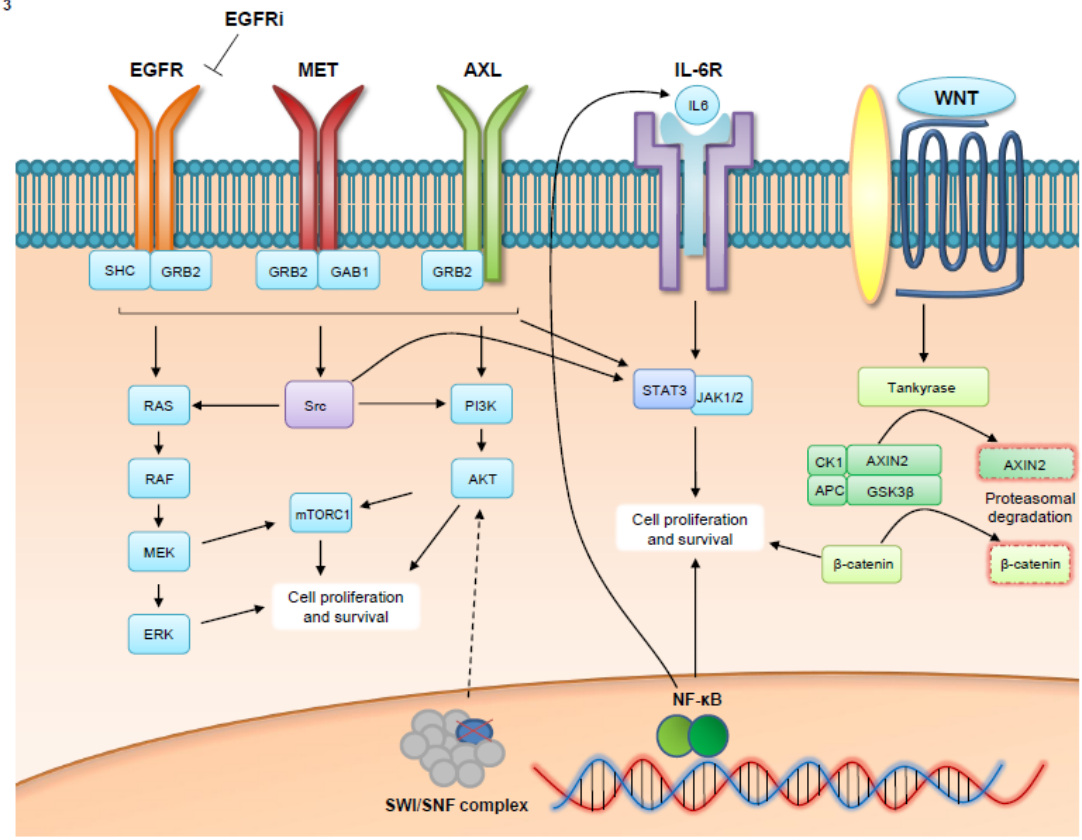
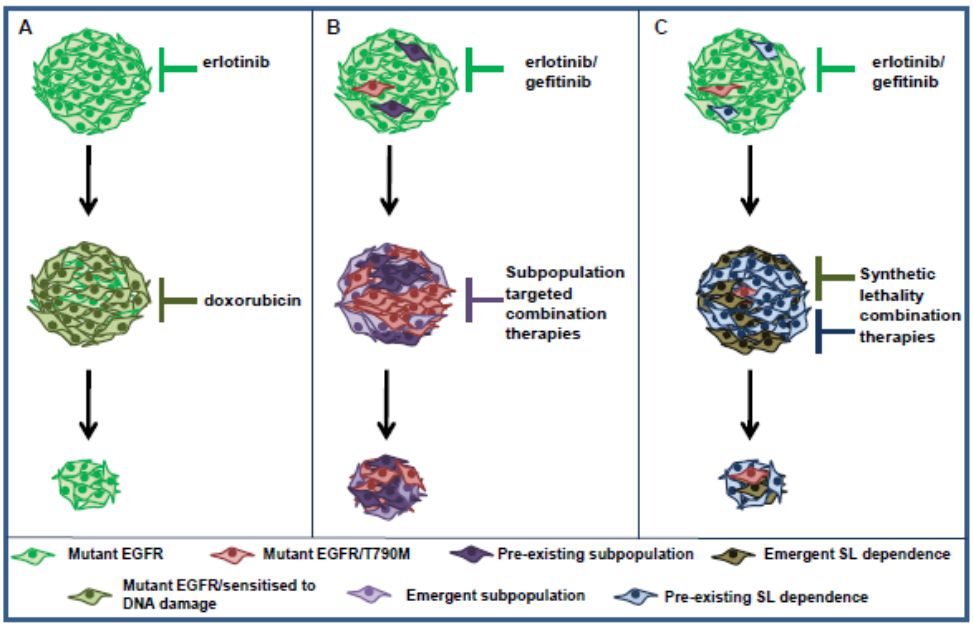
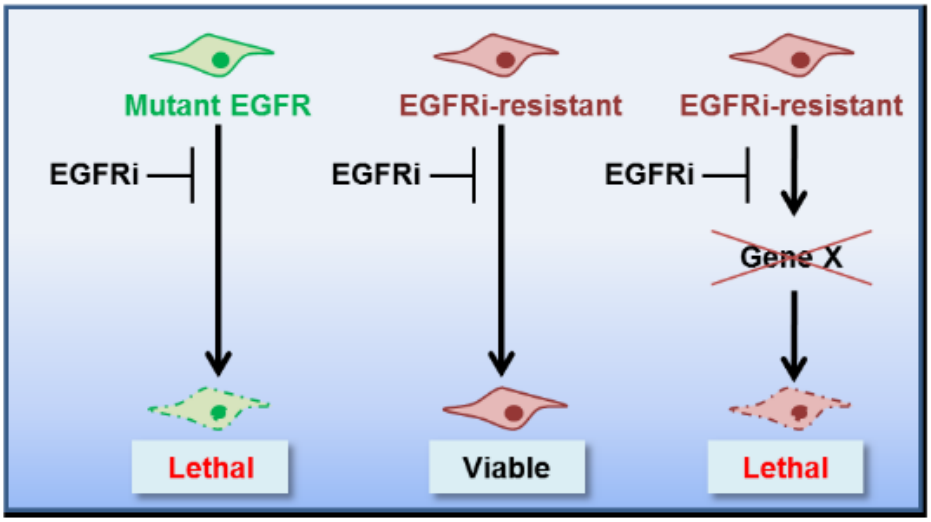


Figure 4





Graphical abstract

Table 1

Table 1. Synthetic lethal interactions identified in EGFR mutant and overexpressing cell line models using functional and profiling screens

Synthetic lethal hit(s)	Model(s)	Screening methodology	Year	Reference
NF-κB pathway	H1650 (ex19del EGFR) EGFRi-insensitive lung cancer cell line	~2000 genes, pooled shRNA screen + erlotinib	2011	[51]
PRKSCH; CD27 signalling	H1975 (L858R, T790M EGFR) acquired EGFRi-resistant lung cancer cell line	Genome-wide pooled shRNA screen + gefitinib	2015	[56]
STAT3; Protein kinase C (PKC); SHC1	A431 (EGFR overexpression) cervical carcinoma cell line	Targeted siRNA screen of 638 "EGFR interactome" genes + drug (erlotinib, panitumumab, CPT11)	2010	[42]
Tankyrase/WNT	H322C (wild-type EGFR) EGFR-insensitive and HCC4006 (ex19del EGFR) EGFRi-sensitive lung cancer cell lines	Genome-wide pooled shRNA screen + gefitinib	2012	[64]
Src family kinases (SFK); AXL; MET; IRS2	PC9 (ex19del EGFR) EGFRi-sensitive and PC9GR (T790M EGFR) gefitinib-resistant lung cancer cell lines	Mass spectrometry-based tyrosine phosphoproteomic profiling of EGFRi-sensitive and resistant cell lines + gefitinib	2014	[46]
CDK6; MARK3; PBK; TBK1; DDR1; EPHA4	H1650 (ex19del EGFR) EGFRi-insensitive lung cancer cell line	~600 kinase genes, pooled shRNA screen and integration with kinome RNAseq expression data	2014	[67]
SWI/SNF subunits: PBRM1, ARID2, ARID1A, ARID1B, SMARCB1	PC9 (ex19del EGFR) EGFRi-sensitive lung cancer cell line	Pooled CRISPR/shRNA screen of ~500 predicted tumour suppressor genes and overexpression screen of ~50 oncogenes, + erlotinib	2017	[75]
MAPK/Rho pathway components: GRB2, SHC1, MK12; PI3K pathway components; CDC37 associated kinases	PC9 (ex19del EGFR) and HCC827 (ex19del EGFR) EGFRi-sensitive lung cancer cell lines	Generation of mutant EGFR interactome using TAP-LC-MS/MS, targeted siRNA screen of 102 "EGFR interactome" genes	2013	[43]

Highlights

- Resistance to EGFR inhibitors in mutant EGFR lung cancer is a major clinical problem
- Synthetic lethal screens have been used to identify targets to overcome resistance
- Translating synthetic lethal hits into novel therapies remains challenging
- Exploiting network biology and tumour heterogeneity may represent potential solutions