Synthetic lethality and anti-cancer drug discovery

Classically-activated oncogene targets have been a mainstay of cancer drug discovery for the past 15 years, but the druggable targets in this category have been largely mined out. Future efforts to drug the cancer genome must take synthetic lethal approaches exploiting vulnerabilities opened up by tumour suppressor mutations. As many cancers do not have enough mutations for immuno-oncology approaches to be fruitful, this area will likely become one of the foremost approaches to cancer drug discovery.

By Jonathan D Moore

The term Synthetic lethality is 70 years old this year, being coined by Theodosius Dobzhansky (**Figure 1**) to describe lethal interactions between different genes in field populations of Drosophila[1]. The concept itself is slightly older, having been described by Calvin Bridges some 25 years earlier[2]. Synthetic lethality in developmental biology can be understood in the context of evolution selecting for resilience against environmental or genetic stress. The developmental biologist and philosopher C.H. Waddington viewed this phenotypic resilience to be distinct from the engineering term of robustness. In 1942, he introduced the concept of canalisation[3] as development proceeding through a river valley enclosed by high ridges, safely guiding the phenotype to its 'fate'. In dynamical systems theory a wild-type like phenotype can be viewed as an attractor, where via interacting positive and negative feedback loops most deviations from the main road still lead to Rome. Looking at PubMed citations (**Figure 2**) the interest in the topic can be seen taking off in the mid 1990s, but the last 10 years have seen an explosion of papers focussing on synthetic lethality in Cancer. How did we get here?

Yeast genetics held high prestige in the late 1980s and early 1990s. Leland Hartwell, Paul Nurse and many others motivated to better understand how cells replicate their DNA and segregate it into two daughters realised the speed at which fundamental advances could be made in this easily manipulated system. A series of clever experiments identified the main players that drove cell cycle progression and also the 'checkpoint' mechanisms that co-ordinated the various steps. Other yeast geneticists were using synthetic lethal screens to place genes in pathways and to understand mechanisms of redundancy. Meanwhile in cancer biology, the concepts of dominantly acting oncogenes and recessive tumour suppressors had been advanced and it was understood that certain tumour suppressors such as ATM and MSH2 had clear functional homologs in yeast. It was also understood that mutations in similar genes conferred striking sensitivities to existing cancer drugs to the affected yeast.

The stage was thus set for Hartwell et al[4] to propose that genetic approaches be integrated into the discovery of anticancer drugs (**Figure 3**). In 1997, functional genetic approaches in mammalian systems were rudimentary: making a knockout in somatic cells or a mouse was labour-intensive and time-consuming. The proposal that model organisms with tractable genetics be used to find targets made sense: conservation of function across biology meant there was a good chance that a mutation in yeast would create similar dependencies on other genes as it might in cancer cells. Genes mutated in cancer provide some sort of proliferation/survival advantage but could also reduce phenotypic resilience, opening up vulnerabilities that untransformed cells do not have. Synthetic lethal screens to find targets exploiting vulnerabilities

Figure 1
Theodosius Dobzhansky
(photograph courtesy of the Rockefeller University Archives)

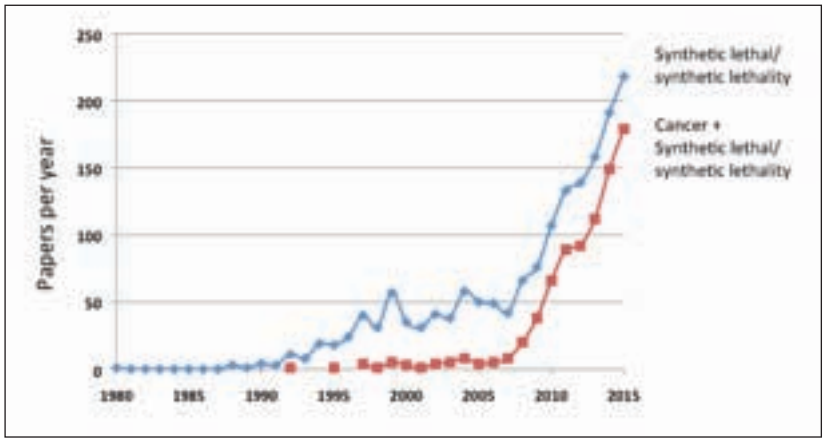

Figure 2: Pubmed citations by year for Synthetic lethality (blue) and synthetic lethality and cancer (red)

created by mutations in tumour suppressor genes were feasible in yeast and Drosophila, but completely unrealistic in mammalian cells.

It is not clear whether there was any significant effort to follow this advice in the pharma industry. This was the dawn of the age of molecular targets

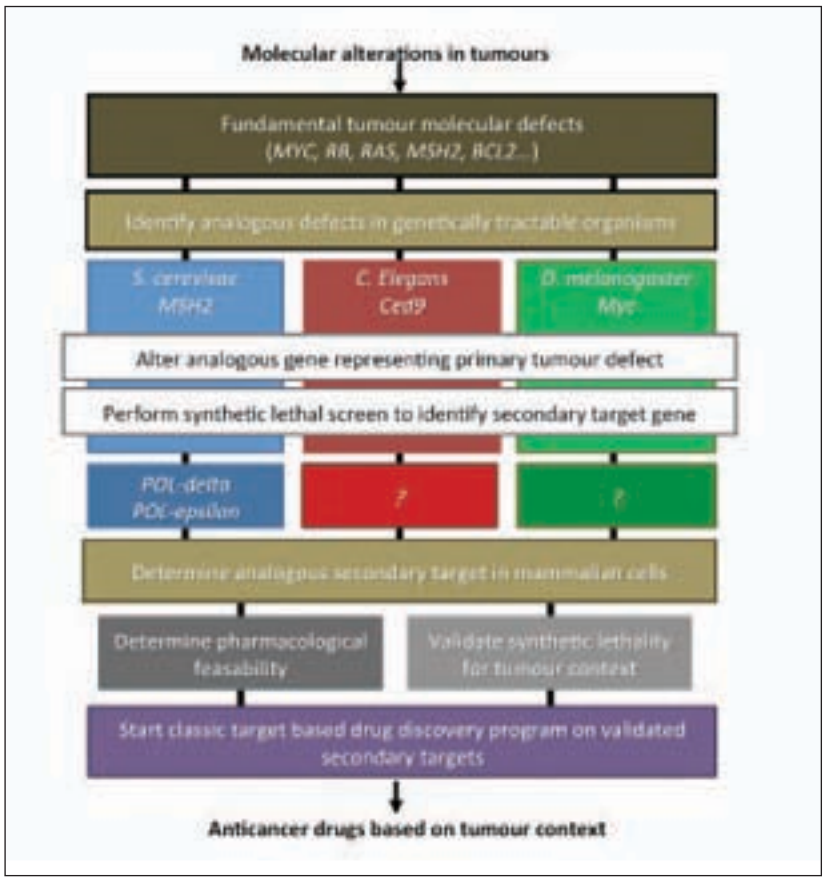

Figure 3: Schematic of synthetic lethal target discovery in model systems *(adapted from reference 4)*

and there were ample dominant gain of function targets to pursue in BCR-ABL, EGFR and HER2 (where an antibody approach had already been fruitful). However, while there was a lack of functional genomic approaches in mammalian cells suitable for screening, screening compound libraries was highly tractable via high throughput screening. Two papers published in 2001 and 2003 reported screens that identified small molecules exhibiting selective toxicity in the KRAS mutant member of an isogenic pair[5,6]. While KRAS is an oncogene rather than a tumour suppressor, oncogenic mutations tend to inhibit its catalytic cycle and direct drugging of KRAS has proved exceptionally challenging.

At this point the way was opening towards functional genomics in human cells with the realisation that short interfering RNAs could fly under the radar of PKR activation and enable the RNA interference mechanism to be exploited in mammalian cells[7]. Pooled retroviral/lentiviral shRNA screening approaches[8] followed soon after in 2004. Such pooled screening approaches collapsed the costs of hypothesis free synthetic lethal screens making them possible (if not advisable) to perform on a genome-wide scale. Initially these screens tracked the abundance of barcodes read on microarrays, but advances in high-throughput DNA sequencing later enabled the actual shRNA sequence to be used as a tracking barcode (**Figure 4**).

PARP inhibitors and BRCA mutant cancers

But what proved to be the big breakthrough for synthetic lethal field came not from a fishing expedition to find new targets but from hypothesis testing. In the early 2000s several industry groups were investigating PARP inhibitors for their ability to inhibit DNA repair and synergise with conventional anticancer cytotoxics. These efforts were built on more than 20 years of academic research. PARP function was known to be vital for base excision repair, the key mechanism for repairing single-stranded breaks. In the absence of PARP activity such lesions persist until repaired by homologous recombination. The tumour suppressors BRCA1 and BRCA2 were known to be required for DNA double-strand break repair by homologous recombination. Two UK-based groups then had the idea of testing whether PARP inhibitors would exhibit enhanced single-agent activity in BRCA mutant cells[9,10]. These cells proved spectacularly more sensitive to PARP inhibitors than normal cells or cancer cells without mutations in this pathway: up to 1,000-fold sensitivity could be demonstrated *in vitro*.

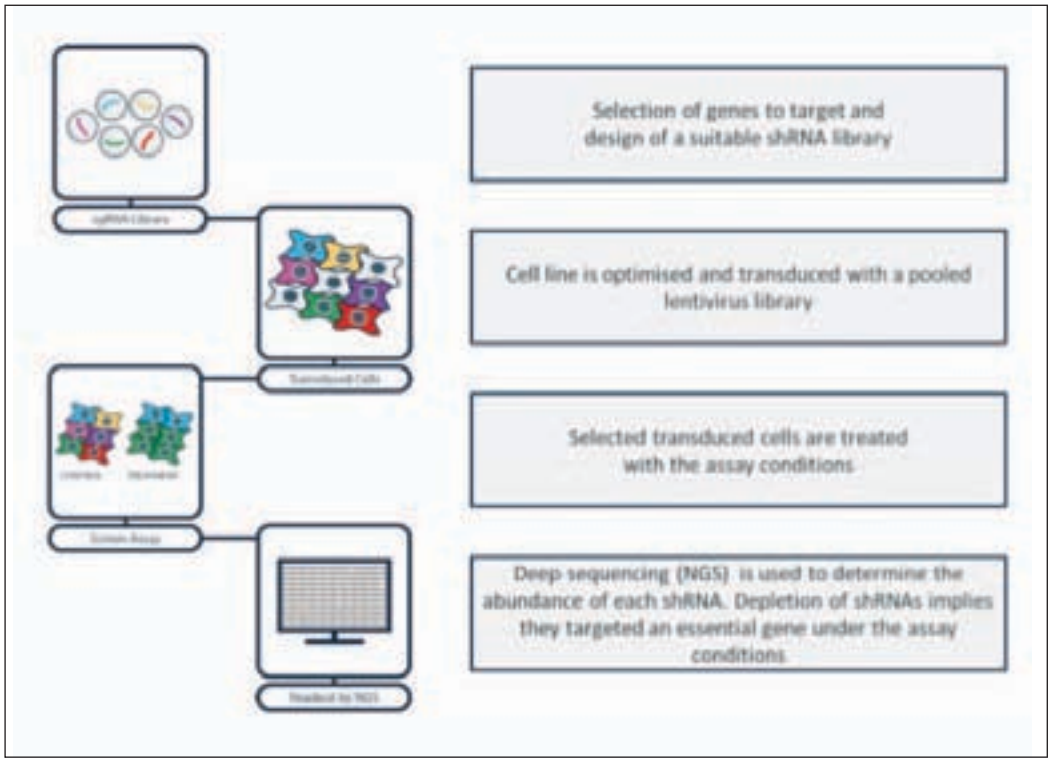

Figure 4
Schematic of shRNA drop out screening. CRISPR screens are essentially performed in the same way

The archetypal PARP inhibitor, olaparib (AstraZeneca) was approved for the therapy of heavily-treated BRCA-mutated ovarian cancers in late 2014, but its path through the clinic was not completely straightforward[11,12] and it has yet to be approved for breast cancer therapy. Phase I trials began in 2005 and the compound found to have activity in advanced breast, ovarian and prostate cancers. In Phase II, activity was seen in ovarian cancer patients with BRCA1 or BRCA2 mutations and also in a number of patients without such mutations. There were initially doubts about the duration of survival benefit, but a subset analysis of the BRCA1/BRCA2 mutated population demonstrated that olaparib treatment made a clear difference to longevity, triggering a pair of Phase III trials starting in early 2013 and approval some 18 months later. One lesson that could be drawn from this is that initial Phase II trials for agents such as olaparib should be run separately in a tightly-defined patient population. Less focused trials aimed at maximising commercial and therapeutic potential should perhaps be run later, at the same time as the Phase III trial in the primary patient group.

Synthetic lethal approaches to p53

BRCA1 and 2 mutations are present in only a relative low fraction of cancers. The tumour suppressor that dominates the cancer driver gene landscape is p53: 42% of all cancers in the TCGA analysis have a TP53 mutation[13], making p53 mutant cancer some 10x more abundant than BRCA1/BRCA2 mutant neoplasms. p53 is a complex protein that acts as a tumour suppressor, and cannot be drugged directly (though a fraction of patients with temperature sensitive p53 mutations could benefit from a drug that stabilised the protein leading to restoration of function and death). So how have synthetic lethal approaches to exploiting TP53 mutations fared?

Hypothesis-testing approaches have been predominant in TP53 synthetic lethal research, in particular the concept of using inhibitors of CHK1 in combination with chemotherapy. This hypothesis rests on the assumption that entry into mitosis with damaged DNA is fatal and that normal cells have redundant pathways to prevent this happening. One pathway involves p53-mediated induction of CDKN2A in response to DNA damage, arresting cell cycle progression by the CDKN1A gene product, p21, binding and inhibiting S-phase promoting CDK/cyclin complexes. The other involves ATR-mediated activation of CHK1 in response to DNA damage, with CHK1 phosphorylating and inactivating the CDC25 phosphatase, which governs activation of M-phase promoting CDK/cyclins. In cancer cells, p53 is often non-functional and such

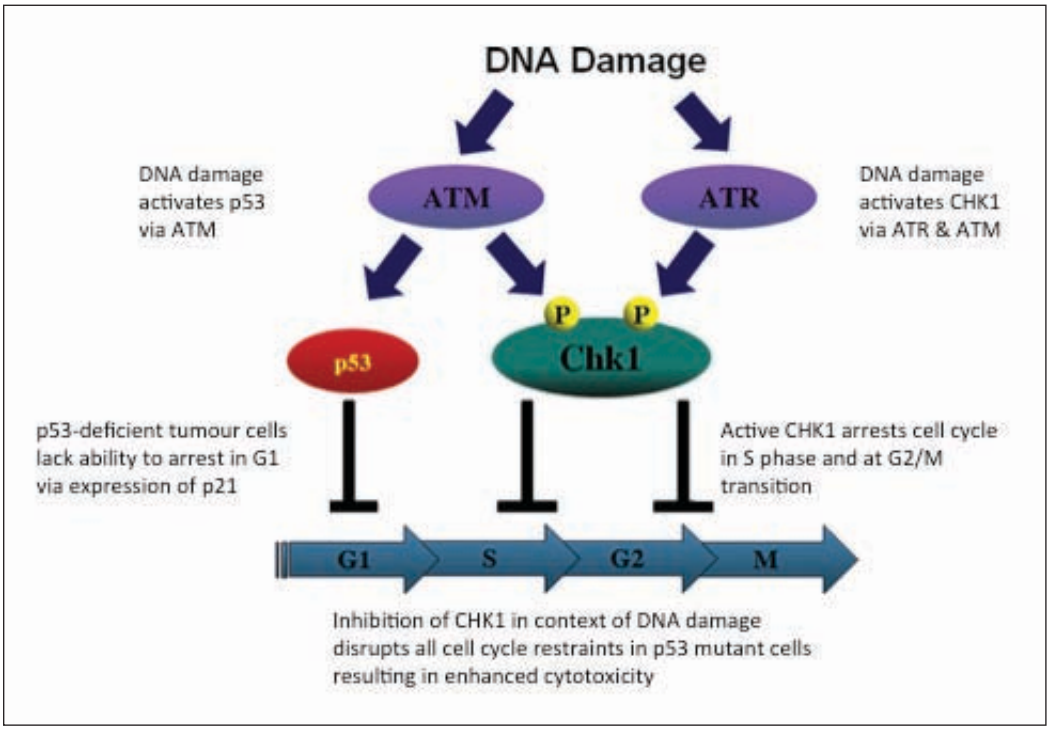

Figure 5: How p53 mutations are hypothesised to sensitise cells to CHK1 inhibitors

cells may rely on CHK1 for survival in the face of DNA damage (**Figure 5**). Many pharma/biotech research programmes have sought CHK1 inhibitors and several compounds have advanced into the clinic, at least one into Phase II. While there is some pre-clinical data to support the hypothesis of using CHK1 inhibitors in TP53 mutant cancers[14], p53 mutations do not appear to be a good biomarker for a cancers response to CHK1 inhibition. The most advanced clinical trials of Chk1 inhibitors now appear focused on BRCA1/2 mutations.

ATR, is also important for the response to DNA damage and several companies have developed ATR inhibitors that have progressed into the clinic. The evidence for synthetic lethality between TP53 mutations and ATR inhibition appears a little stronger than for Chk1 inhibition[15] and one of the Phase II trials for VX-970 has the near universally TP53 mutant ovarian cancer as a focus. However, TP53 mutant cancers are not the only indication under investigation for this compound. The pharma industry's investment in ATR and CHK1 inhibitors could be categorised as a flight to the apparent safety of the familiar: CHK1 and ATR were prominent in the basic mammalian cell cycle research community between 2000-05, so there was good science behind the targets. However, the literature indicates both ATR and CHK1 are essential in mammalian cells and therefore one should have anticipated that the therapeutic window for CHK1 and ATR inhibitors would be narrow at best.

Is there a needle in the KRAS haystack?

RNA interference screens have been a prominent approach to the search for synthetic lethal targets for cancers with KRAS mutations. Given the frequency of KRAS mutations in some cancers with high medical need13 (eg ~40% of colorectal cancers and 25% of lung adenocarcinomas) an effective molecularly targeted agent for these cancers could be a massive therapeutic and commercial success. The obvious strategy of targeting downstream effectors has yet to prove successful, so screens aimed at defining unexpected dependencies make considerable sense. In 2009, two protein kinases were advanced as potential synthetic lethal targets for cells bearing KRAS mutations, STK33 and TBK1. STK33 was identified in an shRNA screen as specifically essential in KRAS-dependent cells, where it acted to prevent BAD induction and apoptosis[16]. However, follow up experiments by another group using siRNA, dominant mutant overexpression and small molecule inhibitors could find no support for the original hypothesis[17]. TBK1 was also identified as a KRAS synthetic lethal target by the same group[18] and again the proposed MOA was that mutant KRAS stimulated pro-apoptotic signalling, which TBK1 was

required to suppress. A detailed mode-of-action study has since been reported on compounds that inhibit TBK1, which claims that autocrine signalling via CCL5 and IL6 maintains the viability of KRAS mutant cells[19]. However, scepticism will still remain regarding these claims until there is independent verification of the results with additional TBK inhibitors.

There have been persistent claims that KRAS mutant cancers exhibit intrinsic genotoxic stress and therefore some prospect of this vulnerability being exploited by checkpoint inhibitors. Dietlein et al[20] recently combined CHK1 inhibitors with inhibitors of the less-prominent kinase MK2, which acts in parallel and applied the combination to a panel of 96 cancer cells cultured *in vitro*. Interestingly, synergy between the CHK1 and MK2 inhibitors was found only in cancers bearing activating KRAS, activating BRAF or loss-of-function mutations in CDKN2A. Moreover, the synergistic effect of CHK1 and MK2 inhibitors was validated in multiple xenograft models.

The RAS biology expert Julian Downward recently summarised the various published screens for RAS synthetic lethality targets, which have highlighted far more candidates than STK33 and TBK1, as being most notable for the low degree of overlap between results[21]. This is not atypical for the technology, as a previous article by Bhinder and Djaballah in this journal has noted[22]: notoriously, three different groups reported results of RNA interference screens searching for host factors required for HIV infection each listing 300 genes. Only three targets were shared, and these did not include the known co-receptor CCR5. There are two basic issues with RNA interference: incomplete knockdown and off-target effects and together these contribute to severe contamination of results with false negatives and false positives.

Can RNA interference find real genetic interactions?

Even Bhinder and Djaballah[22] thought there was value in RNA interference screening. Their article expressed cautious optimism that its flaws could be largely overcome via data transparency and stringent standards for hit nomination. Such standards have arguably been applied to some recent synthetic lethal screens performed to find novel targets for the epigenetic tumour suppressors SMARCA4 and ARID1A, both members of the SWI/SNF complex. Hoffman et al[23] screened an epigenome-focused 17 shRNA/gene library across 58 cancer cell lines and identified SMARCA2, a paralogous protein as being essential for the growth of tumour cells harbouring loss of function mutations in SMARCA4. Similarly, Helming et al[24] reported that ARID1B was selectively required in cancer cells bearing

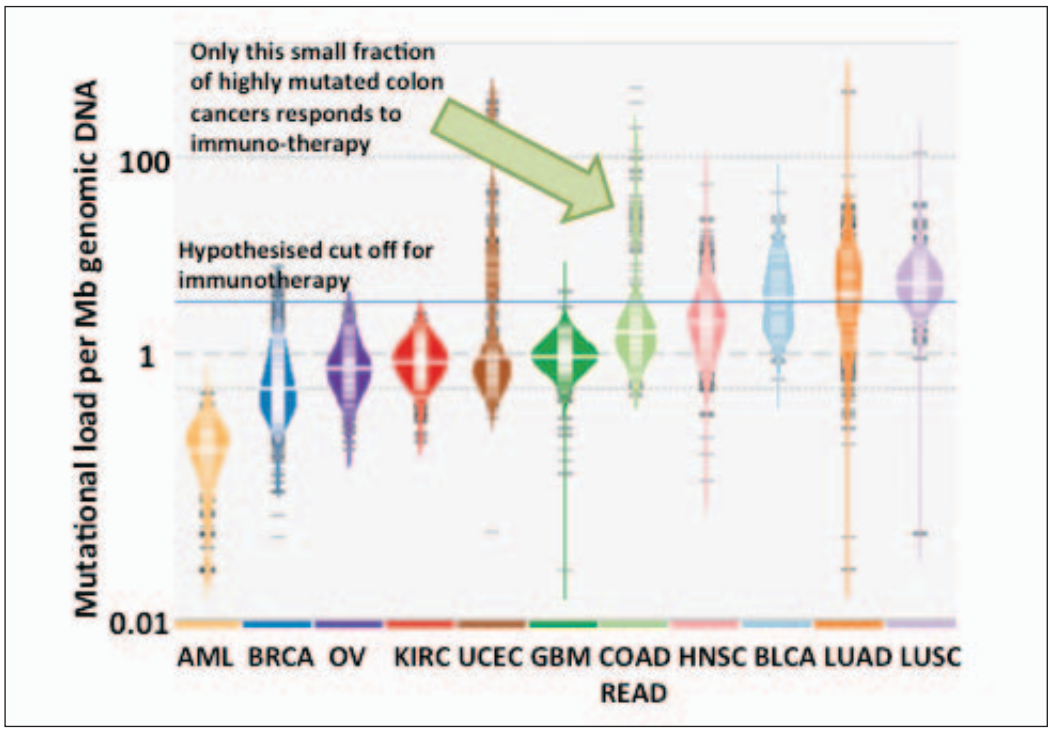

Figure 6: Mutation frequencies in various tumour types. Adapted from TGCA

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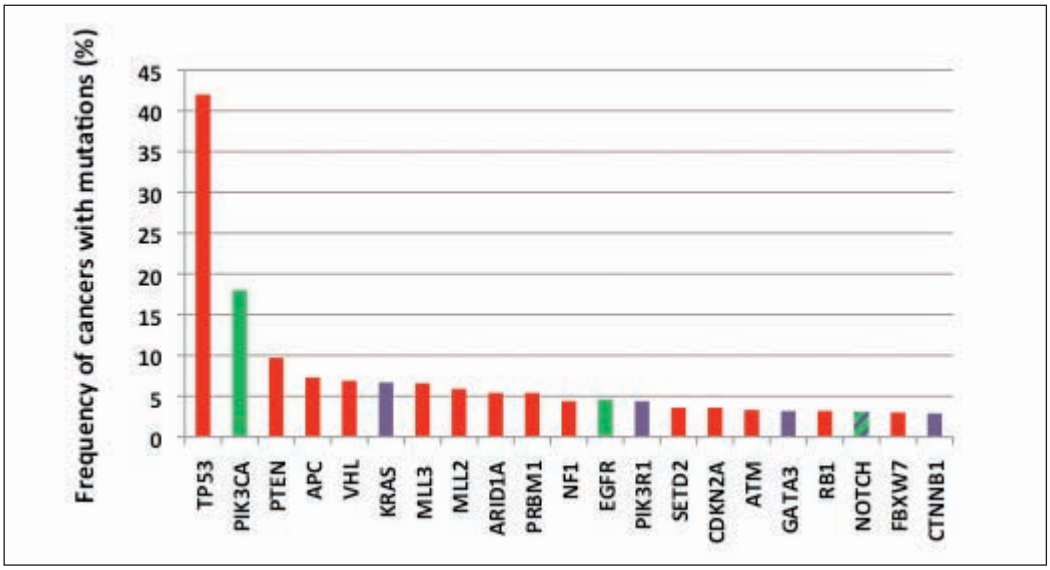

Figure 7: Pan-cancer mutation frequencies in cancer driver genes. Green bars represent druggable oncogene targets, purple bars represent undruggable oncogene targets, red bars represent undruggable tumour suppressor targets

inactivating mutations in ARID1A. These synthetic lethal relationships make perfect sense: ARID1A and ARID1B are mutually exclusive members of the SWI/SNF complex, as are SMARCA4 and SMARCA2. Screens were not really necessary to uncover these dependencies: they were highly predictable based on the biology of the mutated tumour suppressors and these paralogous proteins may not be the best targets for therapies. While it is not unreasonable to say this concept of 'cancer-selective paralog dependency[23]' provides a strategy for targeting tumour suppressors, it does not seem to provide a general solution.

CRISPR: The new hope for synthetic lethality screening

The explosive entry of CRISPR-Cas9-mediated gene editing on to the target identification and validation stage has been recently chronicled in this journal[25] and others[26,27]. Almost as soon as RNA-mediated gene editing was shown to be possible, the previous infrastructure associated with shRNA screening was adapted for large-scale knockout screening. The orthogonal nature and different mode of action of CRISPR-Cas9 perfectly complements RNA interference, but the technology has considerable advantages. Most significantly, gene editing will lead to the complete inactivation of a target, so the issue of incomplete knockdown that plague RNA interference can be overcome. However, a sizeable fraction of Cas9-mediated cuts in genomic DNA are repaired to yield small in-frame insertion and deletion (or substitution) mutants, which means that an edited population will not have a unimodal phenotype and will generally be a mix between homozygous and heterozygous knockouts, mixed with a few unedited cells. This factor confounds negative selection CRISPR screens, limiting the level of drop-out that can be expected when an essential gene is targeted, unless a library is especially designed to target functional domains where any mutation will prove deleterious to function[28]. While screening follow-up pipelines can be devised to cope with this issue, we have yet to see the fruits of such an endeavour. The majority of published CRISPR screens have been in the easier mode of positive selection: identifying drug-resistance genes[29], factors promoting metastasis[30], or phenotypes for which a population of interest can be sorted by flow cytometry[31]. Only one paper to date reports a large CRISPR screen on multiple cell lines[32], but it is clear that many such studies are in the pipeline. Indeed, we are conducting our own at Horizon Discovery.

The five cell lines screened by Hart et al[32] are clearly not sufficient for identifying novel synthetic lethal targets for undruggable tumour suppressors. The use of isogenic cells that only differ in terms of a single mutation of interest is superficially attractive in reducing the size and increasing the clarity of experiments, but suffers from the concern that introducing or deleting a mutation from a cancer cell line in culture does not recapitulate the evolutionary history of tumour cells and so cannot really be expected to reveal every genotype specific vulnerability that would be observed in 'real' cancers.

Instead it seems that screening must be performed in panels of cells. There are good arguments for reducing the size of libraries screened as this allows a corresponding increase in the number of cell lines that can be interrogated.

Validation will be key to sorting the wheat from the chaff in these screens: the Vakoc group's insight that sgRNAs targeted to disrupt important surfaces of proteins drop out far more than their general counterparts provides a natural next-step of follow-up pooled screening. Moreover, knowledge gleaned from the earlier generation of ZFN- and TALEN-mediated gene editing technology has led to the orthogonal approach of CRISPRi, employing enzymatically-inactive Cas9 as an RNA-directed DNA binding protein that can be fused to transcriptional repressors to block transcription[33]. CRISPRi provides a technology with similar features to RNA interference, but acts via blocking RNA expression rather than mediating RNA stability. CRISPRi will prove an invaluable addition to the target validation toolbox.

In conclusion, the prospects for the identification of novel, tractable, synthetic lethal targets are now bright. We can avoid the missteps of the RNA interference era and can expect to go from the current view of targets as through a glass darkly to a clearer vision. While enormous attention is currently focused on immuno-oncology it very much looks like a large fraction of cancers will not have the mutation load that enables their selective elimination by anti-PD1 activated T cells and related approaches (**Figure 6**). Considering pan-cancer mutation frequency, the landscape is dominated by tumour suppressors and undruggable oncogenes (**Figure 7**). The frequently-mutated dominantly-acting druggable targets have been mined out. There is therefore a clear unmet medical need for which drugs versus synthetic lethal targets may provide the solution: let's go and find them! DDW

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