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Combination therapies for personalized cancer medicine

COMBATCANCER

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All cancers arise due to alterations in their genomes. Although insight into the genetic lesions in tumours by genome sequencing does already assist in selecting some drug regimens, it rarely results in disease eradication due to the emergence of drug-resistant clones. More sophisticated combination therapies in which several oncogenic pathways are targeted simultaneously or in a particular sequence are believed to hold more promise. However, at present we are unable to extract and interpret the necessary information from tumours to predict which drug regimen will be most adequate. The genetic make-up of the individual, the heterogeneity of the tumour, epigenetic alterations, cell-of-origin of the tumour, and complex interactions between tumour cells and stromal cells appear important confounding factors influencing response. In addition, we are still ignorant of many of the intricate complexities of signalling networks in cells and how tumours exploit these to acquire drug resistance.
It is the ambition of the team formed by members of the Netherlands Cancer Institute (NKI) and the Cancer Genome Project at the Wellcome Trust Sanger Institute (WTSI) to unravel the genomic and phenotypic complexity of human cancers in order to identify optimal drug combinations for personalized cancer therapy. Our integrated approach will entail (i) deep sequencing of human tumours and cognate mouse tumours; (ii) drug screens in a 1000+ fully characterized tumour cell line panel; (iii) high-throughput in vitro and in vivo shRNA and cDNA drug resistance and enhancement screens; (iv) computational analysis of the acquired data, leading to significant response predictions; (v) rigorous validation of these predictions in genetically engineered mouse models and patient-derived xenografts. This integrated effort is expected to yield a number of combination therapies and companion-diagnostics biomarkers that will be further explored in our existing clinical trial networks.

a. State-of-the-art and objectives

Background and significance - All cancers arise due to the acquisition of somatic mutations in their genomes, which fundamentally alter the function of the protein products of key cancer genes (*1*). Such mutations are responsible not only for the development of the cancer in the first instance but also in maintaining the proliferation status and evasion of cell death that are the hallmarks of cancer (*2*). Over the last decade a number of meticulous studies involving gene resequencing have begun to characterise the genetic changes that occur in cancer, which have revealed the presence of substantial genomic heterogeneity across cancer genomes. To date >400 genes have been identified for which mutations (including somatic coding changes, amplification, deletions and fusion genes) have been causally implicated in cancer (http://www.sanger.ac.uk/genetics/CGP/Census/) (*3*). Moreover, next-generation sequencing of large numbers of tumours across many tissue types are currently underway as part of the International Cancer Genome Consortium (ICGC), and we can expect to have within a decade complete catalogues of somatic mutations in many human cancers (www.icgc.org).

While most of the current treatment regimens for cancer are based on the tissue of origin, the clinical response of cancer patients to treatment with a particular drug is often highly variable. There is a compelling body of evidence, both clinical and experimental, that for an increasing number of drugs used in the clinic the likelihood of a patient's cancer responding to treatment is strongly influenced by alterations in the cancer genome. Arguably the most celebrated example of this has been the use of imatinib, a small molecule inhibitor of the ABL1 tyrosine kinase, to target the fusion protein product of the BCR-ABL translocation seen in chronic myeloid leukaemia (*4*). More recently, the use of EGFR and ALK inhibitors in lung cancer patients whose tumours harbour EGFR mutations and EML4-ALK rearrangements respectively, as well as BRAF inhibitors in melanoma has resulted in significantly improved response rates compared to conventional therapies in those subsets of patients (*5-7*). Although the list of drug-sensitizing mutations is still very low, there are systematic efforts underway world-wide to identify all those genomic alterations that confer sensitivity (or resistance) to cancer compounds in all tissue types. However, it is already becoming apparent from both systematic drug screens as well as clinical experience that large numbers of cancers are intrinsically resistant to single agents, because of co-dependency on multiple signalling pathways, through feedback pathway activation upon drug treatment, or as a result of rapidly acquired resistance despite initial response (*8-12*). Nowhere has this been demonstrated more clearly than in the initial dramatic response seen in BRAF mutant melanoma patients followed by the rapid emergence of drug-resistant disease after treatment with the BRAF inhibitor Vemurafenib (*12*). Both 'intrinsic' and 'acquired' resistance are therefore major clinical challenges and in either case, combinatorial drug modalities that target the relevant activated pathways are an attractive model to overcome resistance.

Experimental approaches and Innovation – Systematic identification of combinatorial drug strategies across multiple cancer types requires large-scale and integrated research programs, utilising state-of-the-art high-throughput chemical screens together with large-scale loss- and gain-of-function retroviral and lentiviral screens and *in silico* modeling. These strategies ought to be explored in both 2- and 3-dimensional (2D/3D) cell culture systems as well as in more complex model organisms, including genetically engineered mouse models (GEMMs) and patient-derived tumour xenograft (PDX) models ('xenopatients') of the relevant cancers. In addition, all models should undergo whole exome sequencing and genome-wide copy number analysis to determine to what extent they recapitulate the mutational landscape in clinical samples. Finally, where possible, clinical samples from patients both pre-treatment and upon development of resistance should be sequenced as a proof-of-concept for any observed mutational profiles that are indicative of response to a combinatorial drug regimen. The goal of this endeavour is the generation of preclinical data that lead to the design of early phase clinical trials to test combinatorial drug hypotheses in cancer patients.

Both the Wellcome Trust Sanger Institute (WTSI) and The Netherlands Cancer Institute (NKI) have proven long-term track records as well as combined expertise in all of the scientific areas that are critical for the successful execution of such a far-reaching research strategy. It constitutes a unique comprehensive research effort to identify drug combinations overcoming intrinsic and acquired resistance in cancer and across a range of experimental cancer models (Figure 1). We propose to focus this effort on 4 main cancer types, based on either their high prevalence, a current lack of any active treatments, and clinical and experimental expertise – namely breast, colorectal, melanoma and thoracic cancers (mesothelioma and small-cell lung cancer).

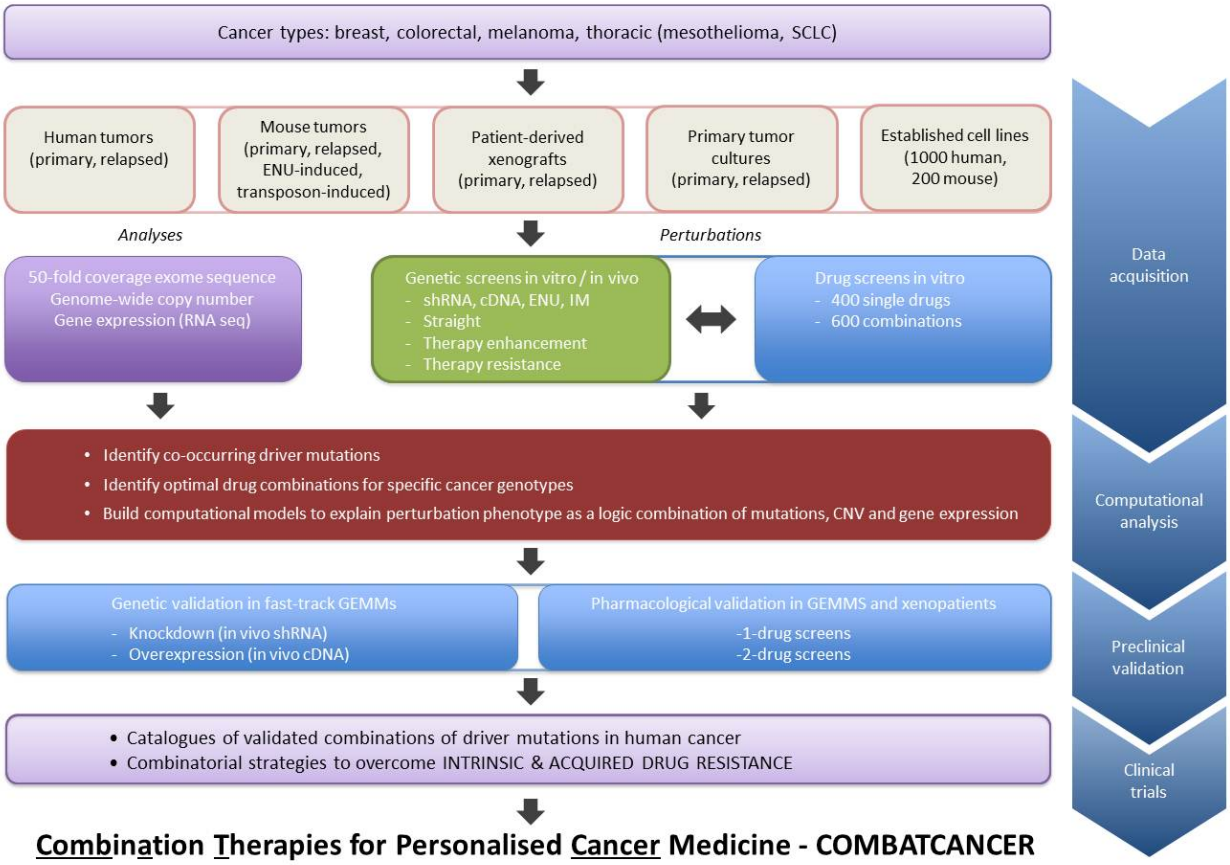

Figure 1: *Schematic of strategic modules of COMBATCANCER (Combination Therapies for Personalized Cancer Medicine): Data Acquisition, Computational Analysis, Preclinical Validation and Clinical trials.*

The WTSI is an internationally-renowned genome sequencing centre, with the capability to define cancer gene variants across large sample sets using automated sequencing and analysis pipelines. It is also home to the largest collection world-wide of genomically-characterised cancer cell lines (>1000). These well-characterised panels of cancer cell lines have been shown to recapitulate many of the known gene-drug associations. Although they fail to capture the 3D complexity of organisms, they are amenable to high-throughput interrogation of hundreds of cancer signalling pathways using either small molecule inhibitors or gene targeting technologies (*13*). At the Sanger Institute we have developed a screen of the effect on viability on 1000 human cancer cell lines treated with cancer drugs (either 400 single agents or 600 2-drug combinations). We are beginning to identify novel mutational events that significantly sensitize to cancer therapeutics (www.cancerRxgene.org) (*14*). These data will identify optimal drug combinations in the context of specific mutations. In addition, we are developing insertional mutagenesis systems in cancer cells and mouse models to enable us to develop acquired drug resistance models across a broad range of cancer types and drug targets. Finally, there is expertise in the use of GEMM of intestinal cancers for the validation of observed drug combinatorial strategies (*15*).

The NKI has a strong track record in functional oncogenomics and mouse models of human cancer. At the NKI, we have developed, or have acquired, retroviral and lentiviral large-scale shRNA (both mouse and human) and ORF cDNA libraries to engineer gain- or loss-of-function gene effects in established cancer cell lines and early passage cells (*8, 16-18*). Such screens are being used to identify gene events that confer sensitivity in the setting of either intrinsic or acquired resistance (enhancer screens) or that are capable of conferring resistance to targeted agents (resistance screens). In addition, in order to capture drug combinatorial effects in physiologically relevant *in vivo* systems we have developed, or have acquired, a large panel of genetically engineered mouse models of breast cancer, melanoma, mesothelioma and small-cell lung cancer (SCLC) (*19-27*) . We have also developed platforms to screen novel drug combinations in xenopatient models. Finally, we have developed expertise in the computational analysis of the large datasets that result from such screens. This allows us to identify statistically significant optimal drug combinations for specific cancer genotypes and to build computational models explaining perturbation phenotypes as a logic combination of mutation, copy number alterations and gene expression (*28*).

Significant synergies and complementarity of the group – The WTSI and NKI participants in this project have a longstanding track record of successful collaboration dating back to 2004. The focus of this collaboration was the generation of genetically engineered mouse models of human cancer, mutational analysis of mouse tumours using insertional mutagenesis screens and next-gen sequencing, and bioinformatics analysis of the resulting data sets. The collaboration has been very intense and involved more than 80 visits and short-term scientific missions between the NKI and the WTSI. In addition to this, frequent joint WTSI-NKI meetings were organised. This collaborative research programme has thus far resulted in 17 joint publications, including 1 *Cell*, 3 *Nature* and 3 *Nature Genetics* papers (*29-35*).

In the context of the current proposal, our existing strong ties will be substantially extended. Indeed, there are multiple ways where the two institutes show complementary expertise and achievements. For example, while WTSI is in the process of analysing and profiling the 1000 tumour cell line collection in the context of targeted therapy, NKI has been developing powerful functional genomic tools (including large-scale shRNA and ORF cDNA libraries) and the expertise to identify novel therapeutic targets, in several tumour settings. Conceivably, the bioinformatics analysis of the mutual hit lists will highlight targets and pathways that are of clinical interest. This will generate broader and more systematic ways to perturb candidate hits and to identify specific synergistic drug interactions. The NKI has generated a large number of validated GEMMs as well as a rapidly expanding collection of PDX models for the tumour types of interest. Together with the newly developed GEMM-ESC technology for rapid validation of candidate cancer genes and drug targets, the GEMMs and PDX models will serve as powerful *in vivo* platforms for validation of novel drug combinations identified by WTSI. In addition, this team will be able to swiftly perform analysis (RNA/DNA) of murine tumours, human tumour xenografts as well as naïve and treatment-resistant tumours.

Goals of research: There is a critical need for the rational design of combinatorial strategies to overcome intrinsic and acquired resistance in cancer using integrated experimental and computational modeling approaches. We propose to identify in a subset of cancers (breast, colorectal, melanoma, mesothelioma and SCLC) experimentally and statistically validated drug combinations that subsequently can be tested in the context of early phase clinical trials in patients with a particular mutation profile.

b. Methodology

Aim 1 - Experimental models of drug resistance to targeted therapies

To generate both intrinsic (Aim1.1) and acquired (Aim 1.2) drug resistance models using high-throughput chemical inhibitor, insertional mutagenesis and functional genetic screens in characterised cancer cell lines.

1.1. Intrinsic drug resistance models

Experimental Approaches – We have established a high-throughput screen of >1000 human cancer cell lines against 400 cancer compounds (either as single agents or as 2-drug combinations) and have developed a computational platform to identify statistically robust correlates of drug response with genomic/ transcriptomic data. This collection of cell line represents the spectrum of common and rare types of adult and childhood cancers of epithelial, mesenchymal and haematopoietic origin. This collection includes approx. 200 melanoma, breast, intestinal, mesothelioma and SCLC cell lines. We used this large panel of cell lines in order to better capture the high degree of genomic diversity in cancer and to identify rare mutant subsets with altered drug sensitivity. All lines were genotyped to exclude the possibility of cross-contamination. All have been subjected to systematic genomic and transcriptional profiling, including sequencing of the full coding exons of 21,416 protein-coding genes and 1,664 microRNA, genome-wide analysis of copy number gain and loss, and expression profiling of 14,500 genes. A complete description of the genetic characterization of cell lines is available online at http://www.sanger.ac.uk/genetics/CGP/CellLines/. For the single drug screen, cells are treated with compound for 72 hours using a 9-point, 256-fold concentration range. We have selected a panel of 400 agents that includes targeted agents, 30 chemotherapeutics, pre-clinical and early phase drugs as well as tool compounds specifically targeting pathways implicated in cancer biology. Effects on cell viability are measured and a curve-fitting algorithm is applied to the raw intensity values to derive a multi-parameter description of drug response, including the half maximal inhibitory concentration (IC50) and the slope of the dose response curve. The drug response data is analysed using both a MANOVA of IC50 values/slope or curve as well as a logic model approach developed in collaboration with the NKI (Lodewyk Wessels). In addition, we have begun to screen the cell line panel against approx. 600 2-drug combinations, using concentration points identified from the single agent screen. This screen will also be extended to a panel of

200 mouse mesothelioma, breast and SCLC cancer cell lines induced in mice with a range of genetic conditional lesions and also targeting different progenitor populations.

This initial analysis can identify statistically significant drug-sensitizing mutations and therefore additionally lead to the identification of subsets of mutated cancer cells that are intrinsically resistant to targeted agents. We propose to identify therapeutic strategies to overcome this resistance by treating these cells with an inhibitor that targets the mutant protein in combination with approx. 100 additional compounds targeting specific cancer pathways. Any synergistic combinations will be confirmed using alternate compounds with similar target specificity. Furthermore, computational models have been developed to identify optimal drug combinations to overcome intrinsic resistance based on mutational data available for each cell line and that can be used to inform the combinatorial drug strategy in Aim 2. The development of these computational models is described in more detail below.

Preliminary data

The high-throughput cancer cell line screen is able to model all of the known drug-sensitizing genotypes with a high degree of sensitivity and specificity and almost 50,000 cell line/drug interactions have been modelled (Figure 1.1). We have confirmed that this screen is able to detect previously identified gene-drug interactions in an entirely unbiased manner, and have already begun to identify entirely novel genomic markers of drug response.

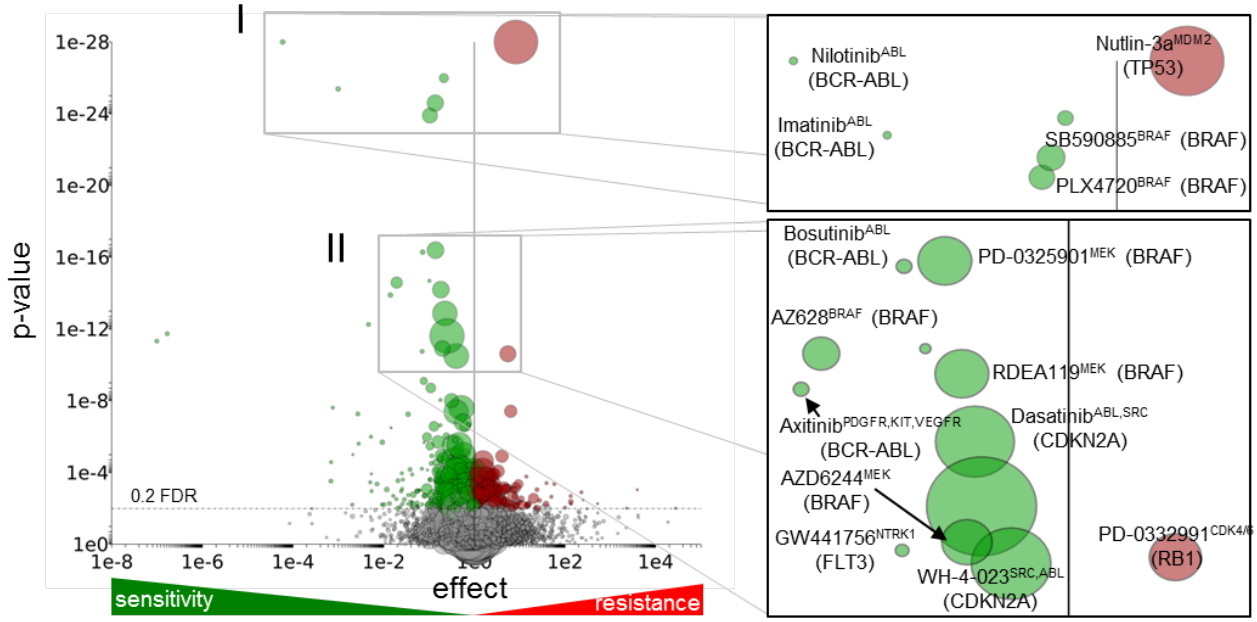

Figure 1.1: Cancer genes modify drug response. *Volcano plot representation of MANOVA results showing the magnitude (effect; x-axis) and significance (p-value; inverted y-axis) of 48,178 cell line/drug combinations. The effect measures the relative difference in the mean IC50 from the wild type to mutant group. Each circle represents a single drug-gene interaction and the circle size is proportional to the number of mutant cell lines. The horizontal dashed line indicates the threshold of statistical significance and significant associations with drug sensitivity or resistance are coloured green and red, respectively. Inserts I and II are magnified views of selected highly significant associations and the drug name, therapeutically relevant drug target(s) (in superscript), and cancer gene (in brackets) is given for each.*

1.2. Acquired drug resistance models

Experimental Approaches – In addition to defining drug combinations to overcome intrinsic resistance in the setting of known drug-sensitizing mutations, we will also use the single agent IC50 data from the 1000 cell line screen to identify sensitive cell line populations that can be used to generate cell line models of acquired drug resistance, and against a variety of genetic backgrounds. Available exome sequence data from clinical samples will be used to ensure that the mutational burden of the cell lines is as close a match as possible. We propose using three complimentary approaches (each with specific advantages and disadvantages) to address this problem. These approaches will comprise (a) serial drug exposure and resistant clone expansion and characterisation (exome sequencing/copy number analysis/gene expression together with functional drug screens), (b) use of insertional mutagenesis models in cancer cells for genome-

wide identification of gain- or loss-of-function events that mediate drug resistance, and (c) infection of sensitive cells with retroviral shRNA and expressed cDNA ORF libraries and characterization of resistant clones following drug selection.

a. *Serial drug exposure* – there is extensive literature pertaining to the characterization of drug-resistant cell line clones to identify clinically relevant resistance mechanisms, and in particular in the setting of resistance to EGFR inhibitors in NSCLC and BRAF inhibitors in malignant melanoma (*11, 36*). Advantage – resistant clones can be characterised and also used as biological resources to explore drug combinations to reverse resistance; disadvantage – the success rate for generation of resistant clones is often low and identification of the underlying mutations is time-consuming.

b. *Transposon-induced drug resistance* – insertional mutagenesis has been used extensively in the field of cancer gene detection in mouse models, where the vector can be engineered to contain transposons capable of generating either gain- or loss-of-function events randomly across the genome. These same vectors can thus be used to carry out forward genetic screens in transfected cancer cells where the detection of statistically enriched sites of transposon insertions in cells that display resistance to a specific drug following activation of the insertional mutagenesis and drug treatment may indicate preferential pathways of resistance that can be targeted for drug combinations (*37*). We will also use a (VBIM) lentiviral insertional mutagenesis screening system to generate drug-resistant cell line clones and pools (*38*). This system randomly integrates a lentiviral promoter into the genome, causing the downstream sequence to be overexpressed. Advantage – the genetic events responsible for resistance should be readily discernible; disadvantage – mechanisms of resistance induced by insertional mutagens may not always be clinically relevant.

c. *Function-based screens* – recent advances in the ability to generate barcoded libraries of shRNA and also open-reading frames (ORF) of kinases have made feasible their use to interrogate resistance mechanisms. To perform shRNA library resistance screens, a similar strategy will be used as for sensitizer screens (Aim 2), except that the cells will be treated with a higher concentration of the inhibitor and for a longer duration. We anticipate that cells bearing shRNA that leads to resistance will take some time to proliferate; optimisation experiments will be performed to determine the best time point to analyse the screen. shRNAs that are enriched in the drug treated sample will identify genes and pathways involved in resistance to the inhibitor. Garraway and colleagues described the use of viral-mediated expression of 597 kinase ORF in a BRAF mutant melanoma cell line to identify MAP3K8 expression as a putative resistance mechanism to BRAF inhibitors, and subsequently confirmed the presence of overexpression in resistant patient samples (*17*). We therefore propose to utilise both RNAi and ORF cDNA expression screens to generate specific resistance models. Advantage – in theory any gene can be targeted for silencing or overexpression; disadvantage – off-target effects of shRNA systems need to (and will) be systematically excluded.

Preliminary results

We have obtained several drug-resistant clones upon long-term treatment of NRAS mutant melanoma cell lines with inhibitors in the MEK-ERK pathway. They show reactivation of several signaling pathways including phospho-MEK. We are currently performing RNA/DNA sequencing and biochemical characterization to dissect the mechanism of resistance.

Anticipated outcomes and potential concerns – The combination of an unbiased 2-drug combination screen incorporating specific inhibitors of cancer pathways together with a statistical approach to identify combinatorial gene interactions in resistant populations will give us a high chance of identifying not only the mutational events responsible for the resistance but also compounds capable of reversing it. To our knowledge, this is the most extensively genomically characterized collection of cell lines in existence, and our ability to analyse all known cancer genes increases the likelihood of identifying clinically relevant resistance mutations. It is feasible that the logic model may identify a resistance mutation that is not currently 'druggable' – depending on whether this event is likely to be either gain- or loss-of-function, we will employ in parallel with the drug screens described above large-scale viral shRNA and overexpression screens in selected panels of cell lines as part of Aim 2.

Aim 2 – Overcoming resistance using combinatorial drug screening and large-scale shRNA loss-of-function screens

To use high-throughput chemical inhibitor drug screens (Aim 2.1) in combination with large-scale retro- and lentiviral shRNA loss-of-function screens (Aim 2.2) in cancer cells to identify combinatorial strategies to overcome intrinsic and acquired resistance.

2.1. Combinatorial drug screening

Experimental Approaches – We have begun to develop a high-throughput combinatorial drug screen in the 1000 cancer cell line collection comprising 100 compounds screened as 2-drug combinations. Combination Index (CI) values may be calculated by finding either the difference or the ratio between the observed and expected proportion of surviving cells. If r_1 and r_2 are the proportion of cells surviving treatment with drugs 1 and 2, respectively, and r_{comb} is the proportion of cells surviving treatment with both drugs, the two CI values may be calculated as follows: $CI = r_{comb} - r_1 \times r_2$. Drug resistant cells (either intrinsically resistant as identified in the primary cell line screen or the acquired drug resistant models) will be treated with both single agents as well as the 2-drug combination screen.

2.2. Function-based large-scale lentiviral shRNA screens

Experimental Approaches – Functional genetic screens provide a powerful tool to identify novel components of signaling pathways and can help to identify mechanisms of drug resistance in preclinical models of cancer (*8, 39*). Retroviral and lentiviral libraries have already been generated by us and others, and are currently being used to enable large-scale shRNA loss-of-function screens in murine and human cells. Thus, the use of these libraries will also enable the detection of specific gene silencing that is capable of reversing intrinsic resistance, either alone or in combination with a targeted agent. These screens are likely to indicate novel combination strategies that again can be confirmed in the cell line 2-drug combination screen prior to exploring in the complex 3D assays detailed below. Understanding the mechanisms of resistance and sensitivity will allow us to anticipate routes to resistance in the clinic and provide an opportunity to develop combination treatment strategies.

We propose to use loss-of-function genetic screens to:

a. *Identify genes that mediate the in vitro drug resistance phenotypes identified in Aim 1.* Cell lines with intrinsic and acquired drug resistance will be used for pooled shRNA screens to identify genes whose depletion reverses intrinsic resistance, either alone or in combination with targeted agents. For this purpose, we have generated several focused lentiviral shRNA libraries targeting all human kinases, chromatin modifying enzymes, deubiquitinating enzymes and DNA damage response factors. These focused libraries increase the likelihood that the screen will lead to identification of useful drug targets. For our pooled screens we use deep sequencing to measure the relative frequency of each shRNA vector in the pool of untreated and drug-treated cells, respectively. We routinely observe >95% correlation between technical replicates, demonstrating that we can accurately measure the abundance of each shRNA vector.

b. *Identify genes that are critical for in vivo survival of untreated and drug-treated cancer cells.* To increase the likelihood of identifying physiologically relevant therapeutic targets, we will launch a program for selective cancer drug target discovery *in vivo*. Building on our broad experience with genome-wide functional screens, we will perform *in vivo* shRNA screens to identify genes essential for proliferation and survival of human and murine cancers transplanted into mice. This strategy will first be applied to melanoma, which can be readily propagated in immunodeficient *NOD;SCID;Il2rg*$^{-/-}$ (NSG) mice (*40*). *In vivo* RNAi depletion screens have also been described for lymphoma and breast cancer, indicating that this strategy can be applied to multiple tumour types (*41, 42*).

c. *Identify proteins whose inhibition enhances drug-induced cytotoxicity.* We propose to transduce drug-sensitive tumour cell lines with the above-mentioned focused lentiviral shRNA libraries. The transduced cell populations will be treated for 3-6 days with an IC20 of the inhibitor (such that most cells survive treatment) after which the abundance of each shRNA vector in the drug-treated and untreated cell populations will be measured by deep sequencing. shRNA vectors that are specifically underrepresented in the drug-treated cells indicate synthetic-lethal shRNAs that enhance drug-induced cytotoxicity; genes targeted by these shRNAs are therefore potential drug targets for use in combination with the inhibitor.

Preliminary results

Drug enhancer screens - As one of the proof-of-concepts, we have been focusing on a small molecule inhibitor of $BRAF^{V600E}$ (Vemurafenib), which has recently shown promising results in clinical trials (*7*), however many patients relapse with drug-resistant tumours after only several months. We are performing large-scale genetic screens to identify shRNAs that enhance the effect of Vemurafenib; genes targeted by these hairpins represent potential drug targets for use in combination with Vemurafenib. Our first screen yielded 24 genes for which at least 2 hairpins were depleted (log2 fold change < -2) in the drug-treated cells compared to the control cells. These genes include *RAF1*, several components of the PI3K signalling pathway, receptor tyrosine kinases, and genes involved in focal adhesion/cytoskeletal signalling pathways.

In vivo dropout screen - We first examined the feasibility of the *in vivo* library screening approach, using a bar code library comprising a large number of unique identifiers, which has been previously constructed by our collaborator Ton Schumacher (NKI) to perform high throughput parallel clonal assays (*43*). Comparison of independent tumours showed a strong overlap of bar codes, demonstrating that the complexity of the library is maintained *in vivo*. In a first *in vivo* screen for melanoma, several cancer-relevant genes emerged as candidates, including *PI4K* for which shRNAs dropped out *in vivo* but hardly *in vitro*, as opposed to BRAF shRNAs, which as expected were lost both *in vitro* and *in vivo* (Figure 2.1).

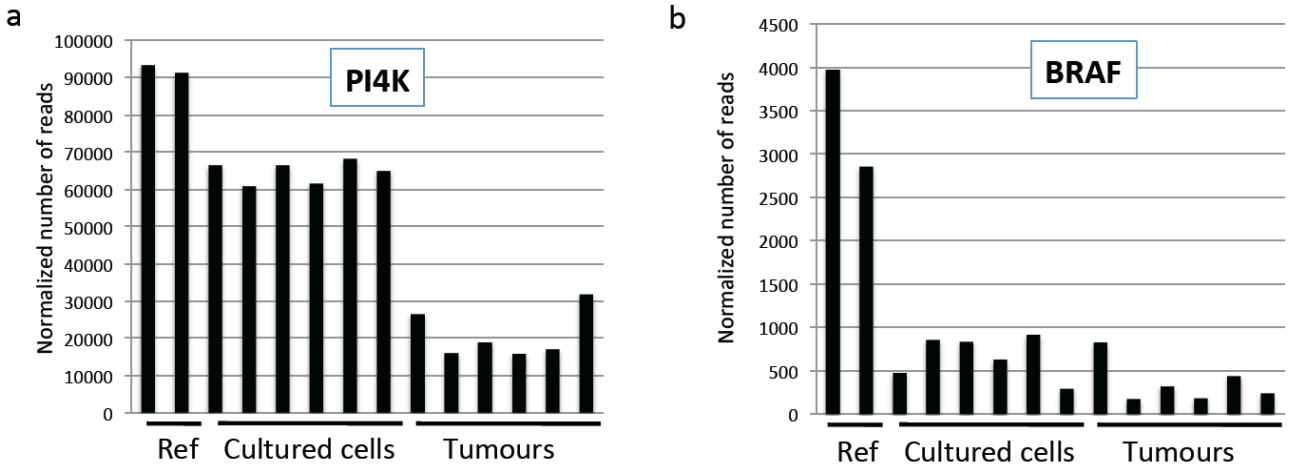

Figure 2.1 *In vivo shRNA dropout screen. Melanoma cells were transduced with a lentiviral shRNA kinome library, pharmacologically selected and either used as a reference, kept in culture in six independent dishes or injected into six immunodeficient mice and allowed to form tumours. After 10 days, gDNA was collected, shRNAs recovered and subjected to deep sequencing. The bar graphs show representative examples of two hits obtained, each with several independent shRNAs (one shown for each gene), and represent the normalized number of reads per sample. a) shRNA targeting PI4KA drops out in all tumours analyzed but not in cultured cells. b) As expected, shRNA targeting BRAF drops out in both cultured cells and in tumours. These results illustrate the feasibility, robustness, specificity and power of in vivo drop out screens.*

Anticipated outcomes and potential concerns – This approach will identify tumour genes that are specifically required for tumour proliferation and/or survival in specific *in vivo*, *in vitro*, tumour, and treatment settings. Furthermore, the shRNA screens are critical in identifying novel combinatorial targets that are not mutated in cancer but are involved in resistance otherwise, for example in paradoxical signaling.

Aim 3 – To build computational models to identify, validate and prioritize optimal drug combinations for specific cancer genotypes

3.1. Identification of combinations of genes that drive tumourigenesis and may affect response to therapy

The sequence of a human cancer genome represents a snapshot of the mutational processes that led to the initiation of the cancer and its evolution. Over the next 5 years an unprecedented number of high-coverage cancer genomes will be made publicly available through the ICGC. For the first time, complete catalogues of somatic mutations for 50 cancer types will be discernible. In most cancers the genetic changes that represent cancer driver mutations are swamped in number by passenger mutations, and identifying the genes that influence patient outcome in response to therapy is greatly complicated by this complexity and by the many different types of genomic lesions that a cancer may acquire (*1*). Exploiting the evolutionary distance

between mouse and human and engineered mouse models of cancer we have performed a large number of forward-genetic screens for cancer drivers. This work initially commenced using retroviruses (*44*) and more recently transposon-mediated insertional mutagenesis and genome sequencing has been employed (*33, 45*). These data have been combined with data from the systematic analysis of human cancer genomes, including whole genome sequencing data, to identify key cancer genes and genetic interactions between these genes (*46*). Much of this progress has been made possible by strong collaborative links between the NKI and the Sanger Institute and we have published over a dozen high-impact publications on this work together (*30, 33, 35, 45-54*). Here we plan to leverage this expertise to identify combinations of mutated genes that may influence response to therapy. Our key objectives are:

a. To systematically analyse the profiles of somatic mutation in cancers sequenced by the ICGC (currently totalling 2449 tumours of 13 types; 25,000 cancers by 2016) to identify mutations that may contribute to tumour development and response to therapy and to release these data via the Catalogue of Somatic Mutations in Cancer (COSMIC) (*55*). BAM files (tumour and normal pairs) from the ICGC portal will be used to call variants using CaveMan and Pindel algorithms. Structural variants will also be called as we have described previously (*32*). Variant calls made in this way will be filtered against other datasets such as the 1000 genomes data (curated at the Sanger) to generate a high confidence set of somatic variant calls. From these data we will identify recurrently mutated genes (*30, 56*).
b. To deploy *Sleeping Beauty* and/or *PiggyBac* insertional mutagenesis to identify combinations of cancer genes mutated in mouse models of lobular breast (*WAPCre;Ecad*$^{f/f}$) (*24*), small cell lung cancer (*Rb*$^{f/f}$*;p53*$^{f/f}$) (*57*), mesothelioma (*Ink4ab*$^{f/f}$*;p19Arf*$^{f/f}$) (*19*), colonic cancer (*Apc*$^{f/f}$) (*58*) and to combine these data with existing datasets we have already generated in models of pancreatic cancer, small intestinal cancer (*48*), melanoma (*59*), T-cell lymphoma and B-ALL (*Tel-AML1*) (*60*). We will generate cohorts of up to 100 animals and controls from each of the models listed above and age animals for tumour formation. Panels of tumours will be collected and archived as paraffin and fresh frozen material to facilitate histopathological analysis and insertion site sequencing as we have described previously (*48*). Commonly targeted genes **(CTGs)** will be identified using the Gaussian Kernel algorithm we have described previously (*61, 62*).
c. To sequence the genomes of spontaneously or chemically induced mouse cancers from the abovementioned models and to analyse the profile of somatic mutations in these cancers using the same approaches as used in objective a. This analysis will harvest the patterns of mutation and identify the somatically mutated genes that have contributed to tumour evolution. We have already applied this approach to compare the genomes of mouse and human breast cancers (*45*) and the genomes of B-ALL tumour from both species. Tumour archives will be generated as described above. Tumours will be graded using ICGC criteria (to assess stroma and cellularity) to select up to 100 tumours from each model for exome sequencing on the Illumina platform. Variants will be validated on the MiSeq/HiSeq platform by exome capture. Structural variants will be validated by PCR.
d. To provide functional interpretation of single driver mutations and co-occurring mutations that identifies optimal drug combinations for specific cancer genotypes:
   Human tumours. Using genes extracted in objectives a and b we will identify statistically significant co-occurring variants, mutually exclusive variants, and variants that show significant enrichment in interaction networks or functional databases. The latter allows the calling of commonly mutated pathways (CMPs). We will adapt approaches we have developed for insertional mutagenesis and copy number alterations for this purpose (*63-65*). For the identification of CMPs we will test for enrichment of mutations in pathway and network neighbourhoods around known oncogenes as defined in COSMIC. To this end we will employ functional networks such as the HumanNet (*66*) as well as gene expression correlation networks derived from large public and private gene expression sets. We expect that statistically significant CMPs will allow mutations in genes not appearing in COSMIC to be linked to known oncogenes, hence expanding functional interpretation of the collected data, and boosting statistical power.
   Mouse tumours. Based on the insertional mutagenesis screen performed in the mouse models selected to match the human tumours we will perform similar analyses. Here the approaches we have developed for detecting CTGs, complex interactions between insertions (co-occurrences and mutual exclusivity) as well as the CTPs will be applied directly. The analysis of mouse whole genome sequencing data will be performed as described for human (above).
   Human-mouse comparative analysis. By performing comparative oncogenomics on the mutational landscapes of human and mouse tumours, we will identify a list of common cancer driver genes. We will also overlay oncogenic driver genes identified in the shRNA loss of function screens (Aim 2). With this analysis we will also examine the fidelity of each of the animal models as a pre-clinical tool for assessing

the efficacy of drug combinations and define a high confidence list of driver genes most likely relevant to the human setting.

3.2. Combination treatments derived from logic rules based on molecular profiles

a. *Logic rules from mutation status.* We will employ integer linear programming (ILP) and dedicated software (IBM ILOG CPLEX Optimization Studio) to identify promising combination treatments based on the mutation status of mutated loci and response data of the 1000 cell line panel. We choose logic modelling as it represents a clear and intuitive formalism, and since it simplifies the process of hypothesis formation. ILP, solved with CPLEX, offers rapid convergence to optimal solutions (the best logic rule) of very large-scale problems. To identify combination treatments we will follow a two-stage strategy. First, we will screen the cell line panel for logic rules that identify sensitive populations with high specificity across the collection of drugs. For example, the logic rule 'IF ERBB2 is mutated OR EGFR is mutated THEN sensitive' employs the mutation status of two loci to characterize cell lines sensitive to the ERBB2 inhibitor, BIBW2992. In the second stage, we will focus on the population of ERBB2 OR EGFR mutated cell lines that remain resistant to BIBW2992. By searching for markers that explain resistance in this population, we can identify putative targets to treat in combination with ERBB2. In this specific case we have identified PIK3CA as resistance marker, suggesting that a combination of BIBW2992 and a PIK3CA inhibitor will further reduce the size of the resistant population.

b. *Logic rules from molecular data.* ILP will also be used to construct more elaborate rules using variables from the gene expression, copy number and mutation data. We will avoid over-training by requiring that 1) the complex models perform significantly better than simple models based on mutation status; 2) by employing cross validation to ensure robust rule identification and 3) by reducing the number of input variables by deriving pathway (instead of single gene) activation. We will achieve this by combining gene expression, copy number and mutation data with pathway topologies as represented in pathways databases such as KEGG, Reactome and PID as well as tools for estimating pathway activity such as SPEED. Based on the pathway activation status, we will follow the two-stage strategy outlined above to derive logic rules that characterize sensitive (first stage) and resistant (second stage) populations based on the activity of pathways rather than the status of a single gene. This allows the identification of combination therapies targeting pathways rather than a single gene.

3.3. Construction of validated and consensus combination treatments.

a. *Validation on combination screens.* Putative combination treatments identified in 3.2 will be reprioritized based on the genomic landscape of human tumours that emerges from 3.1 and then validated in the combination screens described in Aim 1. The reprioritized combination treatments will also be employed to guide the further exploration of the space of combinatorial treatments. We will also feedback the results from validation of the predicted treatment combinations into the ILP framework to increase the robustness of our two-stage approach for predicting putative combination therapies from the single drug screen.
b. *Validation on resistance models via mutations.* From Aim 1 we will obtain models of acquired resistance derived by employing different approaches (drug exposure, insertional mutagenesis and functional genetic approaches). From the latter two approaches we will start out with a sensitive population, such as ERBB2 OR EGFR mutated cell lines that are sensitive to BIBW2992. If resistance screening reveals that activation of the PIK3CA pathway confers resistance, it amounts to confirmation of BIBW2992 and a PIK3CA inhibitor as a combination treatment, i.e. conformation of what was predicted to be an effective combination treatment based on the two-stage analysis on the cell line panel. We will systematically determine the consensus between (i) the combination treatments that have been detected with the two-stage approach and reprioritized based on the human mutational landscape emerging from Aim 1, and (ii) combination treatments that emerge from the insertional mutagenesis and shRNA-based approaches employed in Aim 2. These consensus treatment combinations and their associated companion biomarkers (represented by the logic rules) will inform the in vivo validation screens performed in Aim 4.
c. *Validation on resistance models via expression signatures.* Validation of combination markers based on results of resistance models and application of gene expression signatures to relate screening results to the 1000 cell line panel - From Aim 1 we will also obtain models of acquired resistance induced by drug exposure. In contrast to insertional mutagenesis screens and shRNA screens, the target responsible for the acquisition of resistance cannot readily be derived from the screening results. Also note that a gene that emerges as a resistance marker from an shRNA or insertional mutagenesis screen might not be affected

by mutations in the cell line panel. This prevents direct validation of a finding from the resistance screens in the cell line panel. For these cases we will employ gene expression data to confirm findings from the screens in the cell line panel. More specifically, we will construct gene expression signatures that represent the differential expression between the resistant cells derived from the drug exposure and the original sensitive population. This gene expression signature will be used to validate the finding from the screen in the cell line panel. More specifically, within cell lines resembling the cell line used to derive the resistance model and for treatments similar to the drug employed to induce resistance, we will determine the association of the resistance signature genes with drug resistance as derived in the single drug screens. We have already demonstrated the utility of this approach (Figure 3.1). The group of Rene Bernards at the NKI established that MED12 suppression confers resistance to multiple tyrosine kinase inhibitors in NSCLCs and leads to ERK activation and multi targeted-drug resistance in different cancer types. To further investigate (and confirm) the finding that MED12 suppression confers resistance to cancer drugs targeting the MEK-ERK pathway downstream of RTKs, we turned to the 1000 cell line panel. We showed that a MED12 knockdown gene expression signature is significantly associated with resistance to four MEK inhibitors in 152 RAS or BRAF mutant cell lines from the 1000 cell line panel. Not only did this demonstrate the utility of the screen to uncover mechanisms of resistance, it also showed that gene expression signatures can effectively capture the information required to characterize resistant populations. This approach also allowed the investigation of these resistance mechanisms across a much broader range of cancer types than the tumour type in which the original shRNA screen was conducted. This revealed that melanoma cell lines (mostly BRAF mutants) are highly sensitive to MEK inhibition. In contrast, lung cell lines showed intermediate responses, and pancreatic cell lines were highly resistant (See Figure 3.1).

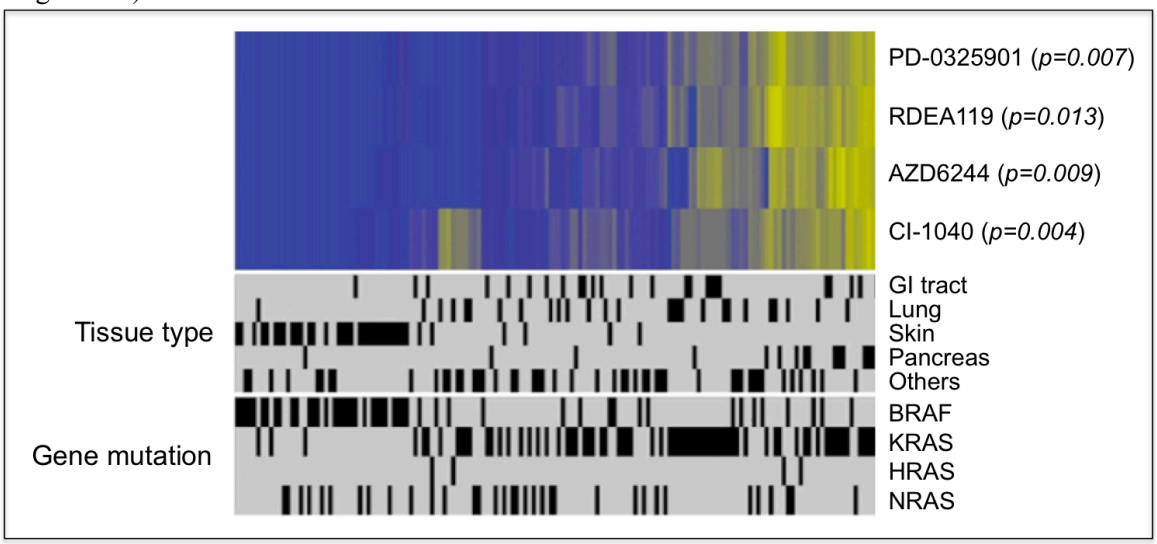

Figure 3.1 *MED12 knockdown gene expression signature predicts drug responses to MEK inhibitors in 152 cell lines of different cancer types harbouring the matching RAS or RAF mutations. High expression of subsets of genes upregulated in the MED12 knockdown signature is significantly associated with higher IC50s for four MEK inhibitors. Across the signature genes, each cell line was scored for the percentage of times it had high expression of the gene as well as resistance to the inhibitor. The heatmap (top panel) of this figure depicts this percentage for each MEK inhibitor (blue: 0%; yellow: 100%). The middle and bottom panel depict the tissue type and RAS/RAF mutation status of the cell lines.*

Anticipated outcomes:

1. Logic rules of mutation markers (single genes and pathways) describing sensitive cell lines with at least 90% specificity for single drugs;
2. A validated computational procedure for identifying combination treatments from single drug screens, mutation and gene expression data;
3. A prioritized list of combination treatments derived by a consensus of 1) the combination treatments extracted by the two-stage approach from the 1000 cell line panel and reprioritized based on the human mutational landscape and 2) combination therapies derived from resistance models through prolonged drug exposure, insertional mutagenesis and shRNA screens.

Aim 4 – Validation of combinatorial strategies using 3D tumour culture models, GEMMs and tumour graft models (NKI, WTSI)

Experimental Approaches – Although established cancer cell lines are tractable models in which to conduct high-throughput combinatorial drug screens, most, if not all of these cell lines have diverged considerably from the original tumours due to continuous selection during decades of *in* vitro culture. Moreover, most *in vitro* culture models do not recapitulate tumour cell-extrinsic factors that contribute to growth and survival of *in situ* cancers. For these reasons, the full complexity of cancer development, progression and therapy response can be studied properly only in the context of intact organisms. We therefore propose to investigate combinatorial treatment modalities identified in the previous aims using a combination of 3D tumour culture models, GEMMs and tumour graft models:

1. 3D tumour culture models:
These models enables us to address concerns as to the biological accuracy of 2D cell-culture systems, namely does growth on plastic accurately model the requirements of cell growth in the cancer patient? We propose to validate combinatorial drug effects in the relevant cancer cell populations using 3D colony formation assays in soft agar and 3D spheroid growth. Cancer cell lines from the tissue types targeted in this study (colorectal, breast, melanoma, mesothelioma and SCLC) will be used to confirm any observed combinatorial effects from the previous large-scale 2D cancer cell line screen. The effect of drug on colony/spheroid number and morphology will be assessed (Image Express, Molecular Devices). 3D cell line cultures enable modelling roles for polarity, matrix interactions, and the impact of mechanical forces not captured in 2D. Thus, there is strong interest in deploying 3D models as drug screening platforms, although current 3D models have limitations in throughput and reproducibility, hampering their implementation for large-scale drug discovery. Our use of both a synthetic scaffold (PuraMatrix) and growth factor-reduced Matrigel create physiologically relevant 3D models that better recapitulate a range of physiologically relevant stromal compliance as compared to rigid tissue culture plastic substrata.

2. GEMMs:
A wide range of GEMMs of human cancer have been developed or imported at the NKI and WTSI, including GEMMs of BRCA-associated hereditary breast cancer (*K14Cre;Brca1$^{f/f}$;p53$^{f/}$* and *K14Cre;Brca2$^{f/f}$;p53$^{f/}$*) (*25, 67*), invasive lobular breast cancer (*K14Cre;Ecad$^{f/f}$;p53$^{f/}$* and *WAPCre;Ecad$^{f/f}$;p53$^{f/f}$*) (*23, 24*), small-cell lung cancer (*Rb$^{f/f}$;p53$^{f/f}$*) (*21, 57*), mesothelioma (*Nf2$^{f/f}$;Ink4a$^{-/-}$; p19Arf$^{f/f}$ and Ink4ab$^{f/f}$;p19Arf$^{f/f}$*) (*19*), melanoma (*TyrCreERT;Braf^{V600E};Pten$^{f/f}$*), and colonic cancer (*Apc$^{f/f}$*) (*58*). In these models the initiating genetic lesions are induced in the cell type that is the relevant cell-of-origin for the type of tumour being modelled. Moreover, tumour initiation and progression occur in an intact, non-mutated microenvironment containing all necessary stromal elements and immune cells required for tumour progression, making these models crucial tools to model human cancer at both the molecular and phenotypic level.

We will use our established and validated GEMMs of human melanoma, breast, colonic and thoracic cancer to validate any combinatorial strategies that are identified in the previous aims. Larger amounts (2-5 grams) of drugs required for *in vivo* studies will be obtained from pharmaceutical companies under standard MTA conditions or ordered from custom synthesis companies. For each combination we will perform dose escalation studies in tumour-bearing mice to determine tumour pharmacokinetics and pharmacodynamics and maximum tolerable dose (MTD). Tumour intervention studies will be typically performed with cohorts of 20-30 mice for each experimental group. Monitoring of tumour growth will be performed using callipers (melanoma and breast cancer models), micro-CT (thoracic cancer models) or endoscopy (colonic cancer models). All dose escalation and intervention studies will be performed in a dedicated Mouse Cancer Clinic established at the NKI, housing specialized intervention rooms and preclinical systems for molecular imaging (bioluminescence/fluorescence) and computerized tomographic imaging (SPECT, CT). The NKI is currently building a new animal facility, which will house an expanded 1400 square metre Mouse Clinic with sufficient capacity for preclinical validation of up to 10 different drug combinations per year.

We will also employ our collection of established GEMMs for validation of candidate cancer genes and drug targets using *in vivo* loss-of-function (LoF) or gain-of-function (GoF) of relevant genes identified in the previous Aims. For accelerated introduction of additional mutant alleles in compound mutant GEMMs we have introduced and optimized methods for rapid and reproducible shuttling of inducible shRNA or conditional cDNA constructs into predefined genomic expression sites in GEMM-derived embryonic stem cells (GEMM-ESCs) via FLP/FRT recombinase-mediated cassette exchange (RMCE) (Figure 4.1) (*26*). For efficient re-derivation of germline-competent ESCs from established GEMMs we employ a serum-free defined culture medium developed by Austin Smith in Cambridge, UK (*68*). Using this approach, we have already established GEMM-ESC lines for our breast and thoracic cancer models and we are currently retrofitting these GEMM-ESC lines with FRT sites in the *Rosa26* and *Col1a1* loci for RMCE-mediated

introduction of additional mutant alleles. The modified GEMM-ESCs can be used for direct production of experimental cohorts of chimeric mice or for production of experimental cohorts of F1 animals by breeding the chimeras with the original GEMM. Since production of novel GEMMs via the GEMM-ESC strategy takes 4-6 months instead of 20-24 months, we will be able to produce at least 5 novel models per year.

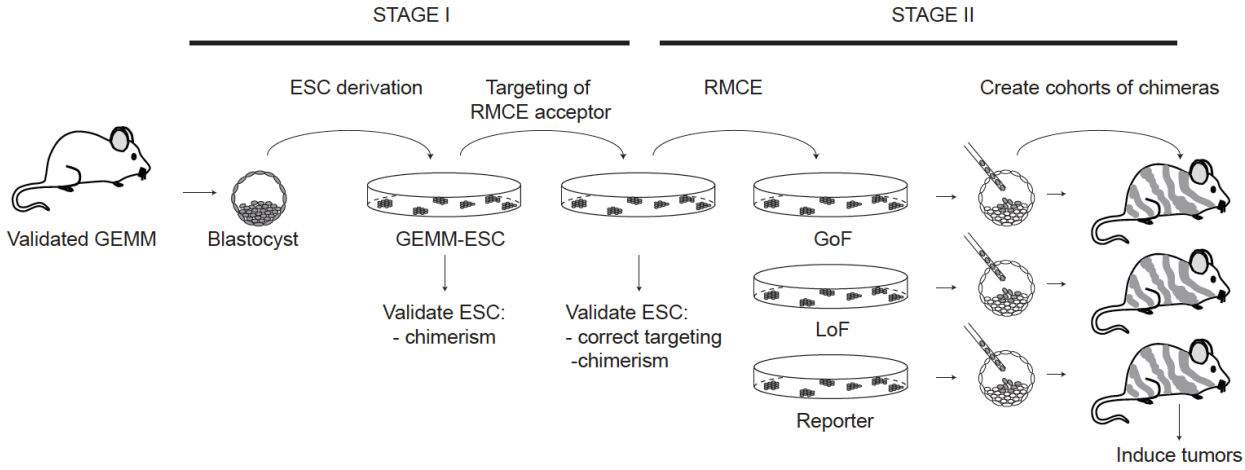

Figure 4.1 *Outline of the GEMM-ESC strategy. Following derivation of embryonic stem cell (ESC) lines from genetically engineered mouse models (GEMMs) of human cancer (Stage I), recombinase-mediated cassette exchange (RMCE) vectors are used to introduce Gain (GoF) or Loss-of-Function (GoF) vectors, or reporters (Stage II).*

3. Tumour allograft models:

A disadvantage of tumour intervention studies in GEMMs is that it often takes several months before "spontaneous" tumours develop. Moreover, several individual tumours per experimental group are required to compensate for the intrinsic inter-tumour heterogeneity in GEMMs. A solution for these complications is the orthotopic transplantation of small tissue pieces from individual GEMM-derived tumours into several syngeneic wild-type immunocompetent recipient mice. The ensuing tumour outgrowths maintain the gene expression profiles and drug responses of the parental tumour. Thus, orthotopic tumour allograft models may be particularly useful for testing various drug regimens on independent outgrowths of the same tumour (*69, 70*). Small tumour fragments of the original GEMM-derived tumours are cryopreserved like cell lines, which permits biobanking of large numbers of primary tumours and molecular characterization to allow preselection of tumours with high expression of the drug target prior to transplantation and initiation of the intervention study. When luciferase or GFP-labelled tumours are used grafting will be performed in genetically modified recipients that express luciferase and GFP in the pituitary under the Growth Hormone promoter in order to prevent tumour rejection through immune responses to GFP or luciferase.

We have already implemented the allograft approach for intervention studies in our breast cancer and mesothelioma GEMMs and we will develop similar orthotopic transplantation strategies for our GEMMS of melanoma, and SCLC (*71*). We will perform whole-exome sequencing and RNA sequencing on the already available collection of cryopreserved tumours in order to be able to preselect tumours for specific intervention studies on the basis of their mutational profile.

4. Patient-derived tumour xenograft (xenopatient) models:

Triple-negative breast cancers (TNBCs), which account for about 15% of all invasive breast cancers, are defined as tumours that lack expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor type 2 (HER2). Consequently, patients with TNBC cannot be treated with endocrine therapy or HER2 targeting therapeutics. To study chemotherapy sensitivity in TNBCs, we have generated a series of PDX models of TNBC by *in vivo* propagation of primary human tumour tissue in immunodeficient mice, using established methods (*72, 73*). Using this approach, we have established BRCA1-proficient TNBC models as well as TNBC models with epigenetic inactivation or genetic mutation of BRCA1. Treatment of these TNBC xenografts with DNA crosslinking agents (cisplatin, melphalan, nimustine) or the PARP inhibitor olaparib showed that BRCA1-deficient TNBCs are very sensitive to these agents but cannot be eradicated and eventually become resistant to all drugs tested so far. These TNBC models offer therefore a unique preclinical *in vivo* platform for testing novel combinations of e.g. PARP inhibitors with other targeted therapeutics such as PI3K pathway inhibitors.

We will use a similar approach to systematically graft fresh biopsies from human melanomas into immune deficient NSG mice without intermediate *in vitro* culturing. We will take advantage of the unique

property of the melanoma/NSG model, which allows for efficient tumour outgrowth from very small numbers of tumour cells, thereby greatly enhancing the success of grafting (*40, 74*). Patient tumour samples obtained before and after therapy will be used. Biopsies will be introduced intradermally or subcutaneously, using protocols that have already been established for melanoma cells in the Peeper lab. Also for colorectal cancers such a xenopatient model has been established recently and used for mouse clinical trials of resistance reversal (*75*).

Anticipated outcomes and potential concerns – The different *in vitro* and *in vivo* validation platforms will allow us to perform:

a) Rapid *in vivo* validation of (combinations of) candidate cancer genes and drug targets in already established and well-characterized GEMMs of human cancer.
b) Combinatorial treatment to avoid or attenuate resistance. Directed by our genomic and transcriptomic analyses and functional screens, we will combine targeted drugs and determine the effect on tumour outcome. We anticipate that our *in vitro* 3D culture models and *in vivo* GEMMs, allograft and xenopatient models will provide powerful and robust validation platforms to address the issues that presently dampen the clinical benefit of targeted therapy.
c) RNA/DNA/(phospho)protein analysis of drug-sensitive and -resistant tumours from GEMMs and xenopatient models. This will provide information for comparative analysis of molecular determinants of therapy response and resistance in established tumour cell lines versus *in vivo* tumours.
d) Tumour clonality analysis. Explanted tumour allografts and xenografts will be transduced during brief *in vitro* culture with a lentiviral bar code library. Upon retransplantation of varying numbers of tumour cells, heterogeneity of the resulting tumour outgrowths will be determined by deep DNA sequencing. In collaboration with Schumacher *et al*. (NKI) we have already successfully set up a bar coding system to trace individual tumour cells during tumour establishment after xenografting (*72*).
e) Tumour clonality analysis in the context of therapy resistance: We will investigate the mechanism of resistance to (combinations of) targeted therapeutics, focusing on clonal selection and adaptation. As an in situ readout for the selection of subclones we will use the "confetti" reporter that permits direct recognition of a limited number of cell clones by the expression of (different combinations of) 4 fluorescent reporters (*76*). This permits monitoring of drug-resistant tumour cell clones as function of their microenvironment. Genetic and genomic analyses of pairs of therapy-sensitive and –resistant tumours will address an important question, namely, how the evolution of resistant tumour cell populations emerges under selective pressure.

Given the multidisciplinary nature of our validation systems, we anticipate that we will be able to thoroughly validate our candidate combinatorial hits, in the best available settings currently available; hence, we do not expect significant problems for this aim.

Aim 5 - To assess the clinical utility of observed combinatorial drug strategies from experimental models

Experimental Approaches – regardless of what experimental model is used to derive a combinatorial strategy to overcome intrinsic or acquired resistance, the final proof of any findings will be in the analysis of patient tumour samples pre-treatment and (in the case of acquired resistance) following the development of drug resistance. To this end, at both the NKI and WTSI clinical patient samples pre-treatment and after the development of resistance are being collected for exome sequencing and genome-wide copy number analysis (Figure 5.1) These trials can be viewed at the NIH clinical trials website (http://clinicaltrials.gov/). The data will be used to validate genetic markers of intrinsic resistance in melanoma to BRAF inhibition as well as mutations that mediate acquired resistance in the models described in the aims above. Candidate resistance genes that are identified from the acquired drug resistance models (either serial pharmacologic exposure, insertional mutagenesis or lentiviral libraries) and that are detected in drug-resistant clinical samples will be prioritised for targeted exome capture and high-coverage (500X) sequencing on the pre-treatment samples in order to identify whether these are present at low levels as subclonal populations that undergo selection during the course of treatment. Confirmation in clinical samples of the existence of pre-existing subclones may indicate that specific drug combinations might prevent the emergence of resistance if used as first-line treatments.

In addition, in the UK the Combinations Alliance has been set up as a joint initiative between the ECMC Network (Experimental Cancer Medicine Centre Network) and Cancer Research UK (CRUK) to

collaborate with pharmaceutical companies to deliver investigator-led combination studies of novel agents. The ECMC comprises a network of 19 Experimental Cancer Medicine Centres across the UK with the aim of accelerating the development of new therapies to bring benefits to patients faster. We propose to use the discoveries of this project to define rational drug combinatorial strategies to take forward into early phase clinical trials through this network and in collaboration with CRUK.

Study	Tumor type(s)	Targeted therapeutic(s)		Chemotherapy combination(s)	Trial Identifier
N11OLM	BRCA-like metastatic solid tumours	Olaparib	PARP inh	melphalan	-
TAMSAR	ER positive metastatic breast cancer	TBD, Tamoxifen	Dual PI3K/mTOR inh, SERM		-
BCCB	1st line metastatic TN breast cancer	Bevacizumab	Anti-VEGF ab	carboplatin, cyclophosphamide, paclitaxel	-
M08AFT	Breast cancer, preoperative study	Tamoxifen, Anastrozole, Fulvestrant	SERM, Aromatase inh, SERD		NCT00738777
M09GSK	Breast cancer	GSK2126458	Dual PI3K/mTOR inh		NCT00972686
M11BRI	Metastatic melanoma	RO5185426	BRAF inh		NCT01307397
X11MDX	Metastatic melanoma	Ipilimumab	Anti-CTLA4 ab		-
MelResist	Metastatic melanoma	Vemurafenib	BRAF inh		-
M09BGJ	NSCLC, bladder, stomach breast cancer	BGJ398	Pan-FGFR inh		NCT01004224
M11LDK	ALK-positive NSCLC + other tumours	LDK378	ALK inh		NCT01283516
ROPETAR	Metastatic RCC	Pazopanib, Everolimus	VEGFR inh, mTOR inh		NCT01408004
BDOCT	Stomach cancer	Bevacizumab, Trastuzumab	Anti-VEGF ab, Anti-ERBB2 ab	docetaxel, oxaliplatin, capecitabine	NCT01359397
M10MKO	Refractory ovarian cancer	MK1775	WEE1 inh	carboplatin	NCT01164995
M08MEK	Melanoma, NSCLC, Colon cancer	RO4987655	MEK inh		NCT00817518
M09GSK	Endometrial cancer, bladder cancer	GSK2126458	Dual PI3K/mTOR inh		NCT00972686
M11TWE	Solid tumours	RO5458640	Anti-TWEAK ab		NCT01383733
M07MKC	Solid tumours	MK1775	WEE1 inh	gemcitabine, cisplatin, carboplatin	NCT00648648
M09DAZ	Solid tumours	AZD4547	Pan-FGFR inh		NCT00979134
M10BEZ	Solid tumours	BEZ235	Dual PI3K/mTOR inh	paclitaxel	NCT01285466
M11RCE	HER3-positive solid tumours	RO5479599, Cetuximab, Erlotinib	Anti-HER3 ab, Anti-EGFR ab, EGFR inh		NCT01482377

Figure 5.1: *Clinical studies of targeted single agents or combinations to be used for tissue collection.*

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c. Resources (incl. project costs) [2]

Summary budget table for the entire grant:

	Cost Category	**Months 1-18**	**Months 19-36**	**Months 37-54**	**Months 55-72**	**Total (72)**
Direct Costs:	*Personnel:*					
	PI[3]	135.000	135.000	135.000	135.000	540.000
	Senior Staff	86.655	91.995	98.210	-	276.860
	Post docs	1.036.510	1.303.022	1.326.396	957.000	4.622.928
	Students					
	ARAs	324.982	384.182	393.416	247.500	1.350.080
	Total Personnel:	1.605.647	1.936.699	1.975.522	1.362.000	6.789.868
	Other Direct Costs:					
	Equipment	811.600		10.000		821.600
	Consumables	1.911.496	810.500	810.500	558.500	4.090.995
	Travel	39.000	47.000	47.000	47.000	180.000
	Publications, etc	3.500	10.000	10.000	10.000	33.500
	Other	19.000	36.000	36.000	36.000	127.000
	Total Other Direct Costs:	2.784.596	903.500	903.500	651.500	5.253.095
	Total Direct Costs:	4.390.243	2.840.199	2.879.022	2.013.500	12.122.964
Indirect Costs (overheads):	Max 20% of Direct Costs	878.049	568.040	575.804	402.700	2.424.593
Subcontracting Costs:	(No overheads)	5.250	5.250	5.250	5.250	21.000
Total Costs of project:	(by year and total)	5.273.540	3.413.489	3.460.076	2.421.450	14.568.557
Requested Grant[4]:	(by year and total)	5.273.542	3.413.489	3.460.076	2.421.450	14.568.557

[2] Adapt to actual project duration.

[3] Please take into account the percentage of your dedicated working time (minimum 30%) to run the ERC funded activity when calculating the salary.

[4] Please make sure that the sums by period and cost category match.

Corresponding Principal Investigator: Anton Berns

	Cost Category	Months 1-18	Months 19-36	Months 37-54	Months 55-72	Total (72)
Direct Costs:	*Personnel:*					
	PI[5]	67.500	67.500	67.500	67.500	270.000
	Senior Staff					
	Post docs (6.5)	435.000	585.000	585.000	585.000	2.190.000
	Students					
	Other (ARA) (3)	150.000	202.500	202.500	202.500	757.5000
	Total Personnel:	625.500	855.000	855.000	855.000	3.217.500
	Other Direct Costs:					
	Equipment	310.000	-	10.000	-	320.000
	Consumables	170.250	313.500	313.500	313.500	1.110.750
	Travel	8.000	12.000	12.000	12.000	44.000
	Publications, etc	2.000	6.000	6.000	6.000	20.000
	WTSI-NKI travel & housing	13.000	24.000	24.000	24.000	85.000
	Total Other Direct Costs:	503.250	355.500	365.500	355.500	1.579.750
	Total Direct Costs:	1.155.750	1.210.500	1.220.500	1.210.500	4.797.250
Indirect Costs (overheads):	Max 20% of Direct Costs	231.150	242.100	244.100	242.100	959.450
Subcontracting Costs:	(No overheads)	5250	5250	5250	5250	21,000
Total Costs of project:	(by year and total)	1.392.150	1.457.850	1.469.850	1.457.850	5.777.700
Requested Grant[6]:	(by year and total)	1.392.150	1.457.850	1.469.850	1.457.850	5.777.700

For the above budget table, please indicate the % of working time the PI dedicates to the project over the period of the grant:	**30%**

Anton Berns (PI) - The Netherlands Cancer Institute
Key team members – 2 members of the team (1 senior postdoc and a staff member responsible for running the knockout service; the latter is on the payroll of the NKI) are responsible for developing the GEMM-ESC technology. They are supported by 2 technicians. 1 postdocs will conduct insertional mutagenesis screens, 1 postdoc and one technician is involved in the in vivo screens (together with a postdoc and technician listed under PI Daniel Peeper), 1 postdoc will execute the ENU screens and 1.5 postdocs will be involved in the validation screens together with a technician. 2 postdocs will be engaged in the bioinforrmatic analysis.
Dr. Jos Jonkers, an independent group leader studying Mouse Models at the NKI, is a critical member of the team and will be responsible for the mouse mammary tumour studies described in this application.
Dr. Lodewyk Wessels, an independent group leader in Bioinformatics and System Biology at the NKI, is an equally essential member of the team and takes responsibility for the computational activities described under Aim 3.

[5] Please take into account the percentage of your dedicated working time (minimum 30%) to run the ERC funded activity when calculating the salary.
[6] Please make sure that the sums by period and cost category match.

Existing resources and infrastructure

A transgenic core facility and mouse clinic. A new animal facility is being build and will come online in 2013. This will provide us with the necessary pace to execute this project. The facility contains fluorescent imaging capabilities, a Cone beam irradiator, and a SPECT-CT scanner. Additional imaging equipment will be obtained through other sources.

High throughput robotic screening facility for shRNA, RNAi, cDNA and compound screening.

Additional infrastructure and equipment

To execute this project we need to substantially expand our capacity to produce mice from manipulated GEMM-ESC lines. This will require the addition of two micro-injection set-ups at €150k each and a computer (€ 10K/pc) in year 1 and 4.

Principal Investigator: Michael Stratton

	Cost Category	Months 1-18	Months 19-36	Months 37-54	Months 55-72	Total (72)
Direct Costs:	*Personnel:*					
	PI	-	-	-	-	-
	Senior Staff (Bioinformatician)	86,655	91,995	98,210	-	276,860
	Post docs (2)	163,255	173,011	184,698	-	520,964
	Students	-	-	-	-	-
	Other (ARA)	64,991	68,341	72,958	-	206,290
	Total Personnel:	314,902	333,347	355,866	-	1,004,114
						-
	Other Direct Costs:					-
	Equipment	501,600	-	-	-	501,600
	Consumables	368,230	32,400	32,400	-	433,030
	Travel	13,500	13,500	13,500	13,500	54,000
	Publications, etc	-	-	-	-	-
	Other	-	-	-		-
	Total Other Direct Costs:	883,330	45,900	45,900	13,500	988,630
	Total Direct Costs:	1,198,231	379,247	401,766	13,500	1,992,744
Indirect Costs (overheads):	Max 20% of Direct Costs	239,646	75,849	80,353	2,700	398,549
Subcontracting Costs:	(No overheads)	-	-	-	-	-
Total Costs of project:	(by year and total)	1,437,877	455,096	482,119	16,200	2,391,293
Requested Grant[2]:	(by year and total)	1,437,877	455,096	482,119	16,200	2,391,293

For the above budget table, please indicate the % of working time the PI dedicates to the project over the period of the grant:	**30 %**

Michael Stratton (PI) – Wellcome Trust Sanger Institute
Key team members - Senior Bioinformatician (development of analysis pipeline of ICGC genome data in COSMIC), 2 post-doctoral fellows (follow up observed combinatorial hits from cell line screen), advanced research assistant (to expand 2-drug screen across 1000 cell line panel).
Dr Ultan McDermott, an independent group leader in the WTSI, and Dr Mathew Garnett (senior staff scientist) are critical members of the team and will be responsible for the cell line screens in the project.

Existing resources and infrastructure

a. High-throughput 1000 cancer cell line drug screen – automated platforms to enable single and 2-drug combinations in 2-D cell line culture and informatics pipelines to enable data analysis,
b. Genetically characterized collection of 1000 cancer cell lines (exome sequenced, genome-wide copy number and gene expression data),
c. Database infrastructure for data storage and analysis,
d. Illumina HiSeq2000 next-generation sequencing and analysis pipelines (exome, genome and RNA-seq).

Additional infrastructure and equipment

Image Express (Molecular Devices) – automated high-content analysis of drug effect in 3D culture systems.

Biomek Fxp liquid-handling platform (Beckman Coulter) – to enable high-throughput 2-drug combination screens in 2D and 3D cell line culture settings.

Principal Investigator: Daniel Peeper

	Cost Category	Months 1-18	Months 19-36	Months 37-54	Months 55-72	Total (72)
Direct Costs:	*Personnel:*					
	PI[7]	67.500	67.500	67.500	67.500	270.000
	Senior Staff					
	Post docs (4)	275.000	372.000	372.000	372.000	1.391.000
	Students					
	Other (ARA)	67.500	67.500	67.500	67.500	270.000
	Total Personnel:	410.000	507.000	507.000	507.000	1.931.000
	Other Direct Costs:					
	Equipment					
	Consumables	136.500	245.000	245.000	245.000	871.500
	Travel	4.000	8.000	8.000	8.000	28.000
	Publications, etc	1.500	4.000	4.000	4.000	13.500
	WTSI-NKI (travel + housing)	6.000	12.000	12.000	12.000	42.000
	Total Other Direct Costs:	148.000	269.000	269.000	269.000	955.000
	Total Direct Costs:	558.000	776.000	776.000	776.000	2.886.000
Indirect Costs (overheads):	Max 20% of Direct Costs	111.600	155.200	155.200	155.200	577.200
Subcontracting Costs:	(No overheads)					
Total Costs of project:	(by year and total)	669.600	931.200	931.200	931.200	3.463.200
Requested Grant[8]:	(by year and total)	669.600	931.200	931.200	931.200	3.463.200

For the above budget table, please indicate the % of working time the PI dedicates to the project over the period of the grant:	**30 %**

Daniel Peeper (PI) - The Netherlands Cancer Institute
Key team members – 2 post-doctoral fellows for *in vitro* screens, 1 for identifying genes mediating drug resistance and 1 for proteins whose inhibition enhances drug-induced cytotoxicity. Both will also be responsible for target validation and contribute to preclinical follow up. 1 post-doctoral fellows for *in vivo* screens for identifying critical fitness (proliferation/survival) genes in untreated tumours and for cancers grown in mice that are exposed to single drugs (in this latter part of the project also a postdoc listed under PI Anton Berns will participate). Also these postdocs will participate in validation and preclinical follow-up studies. 1 post-doctoral fellow and 1 technician (together with a technician listed in the budget of Anton Berns) for the xenopatient platform; the technicians will coordinate with the clinicians, and perform the grafting, while the postdoc will perform the functional studies.

<u>Existing resources and infrastructure</u>

[7] Please take into account the percentage of your dedicated working time (minimum 30%) to run the ERC funded activity when calculating the salary.
[8] Please make sure that the sums by period and cost category match.

NKI has a full-genome lentiviral shRNA library available and we have already generated several focused sublibraries targeting all human kinases, chromatin modifying enzymes, deubiquitinating enzymes, and DNA damage response factors.
Xenopatient studies will be carried out in the animal facility that will be expanded next year. Fresh biopsies from human tumours will be systematically grafted into immune deficient (NSG) mice without intermediate *in vitro* culturing.
NKI has a high throughput robotic screening facility for shRNA, siRNA, cDNA and compound screening.
NKI has an operational facility for next generation sequencing and bioinformatic support for analysis.

Additional infrastructure and equipment
-

Principal Investigator: David Adams

	Cost Category	Months 1-18	Months 19-36	Months 37-54	Months 55-72	Total (72)
Direct Costs:	*Personnel:*					-
	PI	-	-	-	-	-
	Senior Staff	-	-	-	-	-
	Post docs (2)	163,255	173,011	184,698	-	520,964
	Students	-	-	-	-	-
	Other (ARA)	64,991	68,341	72,958	-	206,290
	Total Personnel:	228,247	241,352	257,656	-	727,255
						-
	Other Direct Costs:					-
	Equipment	-	-	-	-	-
	Consumables	1,236,516	219,600	219,600	-	1,675,716
	Travel	13,500	13,500	13,500	13,500	54,000
	Publications, etc	-	-	-	-	-
	Other	-	-	-	-	-
	Total Other Direct Costs:	1,250,016	233,100	233,100	13,500	1,729,716
	Total Direct Costs:	1,478,263	474,452	490,756	13,500	2,456,971
Indirect Costs (overheads):	Max 20% of Direct Costs	295,653	94,890	98,151	2,700	491,394
Subcontracting Costs:	(No overheads)	-	-	-	-	-
Total Costs of project:	(by year and total)	1,773,915	569,342	588,907	16,200	2,948,365
Requested Grant:	(by year and total)	1,773,915	569,342	588,907	16,200	2,948,365

For the above budget table, please indicate the % of working time the PI dedicates to the project over the period of the grant:	**30 %**

David Adams (PI) – Wellcome Trust Sanger Institute
Key team members - 2 post-doctoral fellows (for mouse model experiments), advanced research assistant (to co-ordinate the sequencing of mouse tumours and tumour collection and archiving).

Existing resources and infrastructure

a. Mouse models of cancer housed in a state of the art 25,000 cage animal facility.
b. Illumina HiSeq2000 next-generation sequencing and analysis pipelines (exome, genome and RNA-seq).
c. Database infrastructure for data storage and analysis.

d. Ethical and security-sensitive issues

ETHICS ISSUES TABLE

Areas Excluded From Funding Under FP7 *(Art. 6)*

(i) Research activity aiming at human cloning for reproductive purposes;

(ii) Research activity intended to modify the genetic heritage of human beings which could make such changes heritable (Research relating to cancer treatment of the gonads can be financed);

(iii) Research activities intended to create human embryos solely for the purpose of research or for the purpose of stem cell procurement, including by means of somatic cell nuclear transfer;

All FP7 funded research shall comply with the relevant national, EU and international ethics-related rules and professional codes of conduct. Where necessary, the beneficiary(ies) shall provide the responsible Commission services with a written confirmation that it has received (a) favourable opinion(s) of the relevant ethics committee(s) and, if applicable, the regulatory approval(s) of the competent national or local authority(ies) in the country in which the research is to be carried out, before beginning any Commission approved research requiring such opinions or approvals. The copy of the official approval from the relevant national or local ethics committees must also be provided to the responsible Commission services.

	Research on Human Embryo/ Foetus	YES	Page
	Does the proposed research involve human Embryos?		
	Does the proposed research involve human Foetal Tissues/ Cells?		
	Does the proposed research involve human Embryonic Stem Cells (hESCs)?		
	Does the proposed research on human Embryonic Stem Cells involve cells in culture?		
	Does the proposed research on Human Embryonic Stem Cells involve the derivation of cells from Embryos?		
	I CONFIRM THAT NONE OF THE ABOVE ISSUES APPLY TO MY PROPOSAL	YES	

	Research on Humans	YES	Page
	Does the proposed research involve children?		
	Does the proposed research involve patients?		
	Does the proposed research involve persons not able to give consent?		
	Does the proposed research involve adult healthy volunteers?		
	Does the proposed research involve Human genetic material?	YES	xxxx
	Does the proposed research involve Human biological samples?	YES	xxxx
	Does the proposed research involve Human data collection?	YES	xxxx
	I CONFIRM THAT NONE OF THE ABOVE ISSUES APPLY TO MY PROPOSAL		

	Privacy	YES	Page

	Does the proposed research involve processing of genetic information or personal data (e.g. health, sexual lifestyle, ethnicity, political opinion, religious or philosophical conviction)?		
	Does the proposed research involve tracking the location or observation of people?		
	I CONFIRM THAT NONE OF THE ABOVE ISSUES APPLY TO MY PROPOSAL	YES	

	Research on Animals[9]	**YES**	**Page**
	Does the proposed research involve research on animals?	YES	xxx
	Are those animals transgenic small laboratory animals?	YES	xxx
	Are those animals transgenic farm animals?	YES	xxx
	Are those animals non-human primates?		
	Are those animals cloned farm animals?		
	I CONFIRM THAT NONE OF THE ABOVE ISSUES APPLY TO MY PROPOSAL		

	Research Involving non-EU Countries (ICPC Countries[10]**)** [11]	**YES**	**Page**
	Is the proposed research (or parts of it) going to take place in one or more of the ICPC Countries?		
	Is any material used in the research (e.g. personal data, animal and/or human tissue samples, genetic material, live animals, etc) : a) Collected in any of the ICPC countries?		
	b) Exported to any other country (including ICPC and EU Member States)?		
	I CONFIRM THAT NONE OF THE ABOVE ISSUES APPLY TO MY PROPOSAL	YES	

	Dual Use	**YES**	**Page**
	Research having direct military use		
	Research having the potential for terrorist abuse		
	I CONFIRM THAT NONE OF THE ABOVE ISSUES APPLY TO MY PROPOSAL	YES	

If any of the above issues apply to your proposal, you are required to complete and upload the 'B1_Ethical Issues Annex' (template provided).

Without this Annex, your application cannot be properly evaluated and even if successful the granting process will not proceed.

[9] The type of animals involved in the research that fall under the scope of the Commission's Ethical Scrutiny procedures are defined in the Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes Official Journal L 358 , 18/12/1986 p. 0001 - 0028

[10] In accordance with Article 12(1) of the Rules for Participation in FP7, 'International Cooperation Partner Country (ICPC) means a third country which the Commission classifies as a low-income (L), lower-middle-income (LM) or upper-middle-income (UM) country. Countries associated to the Seventh EC Framework Programme do not qualify as ICP Countries and therefore do not appear in this list.

[11] A guidance note on how to deal with ethical issues arising out of the involvement of non-EU countries is available at: ftp://ftp.cordis.europa.eu/pub/fp7/docs/developing-countries_en.pdf

Please see the Guide for Applicants for the Synergy Grant 2012 for further details and the Participant Portal http://ec.europa.eu/research/participants/portal/ for further information on how to deal with Ethical Issues in your proposal.

If your proposal is security-sensitive, complete and upload Annex 5 (template provided).