MEDICAL UNIVERSITY OF VIENNA

Ubiquitin, signalling and cancer vulnerabilities

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Submitted by

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Declaration

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- Section *2.1* partially covers the publication "A chemical-genetic screen reveals a mechanism of resistance to PI3K inhibitors in cancer" (Nat Chem Biol. 2011 Sep 25;7(11):787-93. doi: 10.1038/nchembio.695) (Muellner et al, 2011) and partially contributed to the manuscript "TOPS: A versatile software tool for statistical analysis and visualization of combinatorial gene-gene and gene-drug interaction screens" (Muellner et al, 2013). Here, Markus K Muellner, **Iris Z Uras**, Nils Craig-Mueller and Sebastian MB Nijman set up the Luminex multiplexing assay. **Iris Z Uras**, Markus K Muellner and Bianca V Gapp created and characterized the isogenic MCF10A cell lines. **Iris Z Uras** generated and characterized the isogenic HeLa cell lines. **Iris Z Uras** created the datasets of the SMARTpool kinase siRNA library genetic screens, analysed the Luminex data and performed wetlab validation experiments. Markus K Muellner created the dataset of the chemical screen and **Iris Z Uras** and Markus K Muellner performed wetlab validation experiments. Jacques Colinge and Gerhard Duernberger designed the analysis platform and database infrastructure for the genetic and chemical screens. Gerhard Duernberger and Markus K Muellner analysed the screening data and wrote R code to identify hits. Markus K Muellner performed the majority of experiments to decipher the resistance mechanism to PI3K inhibitors, while Claudia Kerzendorfer, Michal Smida, Hannelore Lechtermann and Sebastian MB Nijman performed and helped with additional experiments.

- Section *2.2* describes the publication "Ubiquitin-specific protease 4 inhibits mono-ubiquitination of the master growth factor signaling kinase PDK1"

(PLOS ONE. 2012;7(2):e31003. doi: 10.1371/journal.pone.0031003) (Uras et al, 2012). **Iris Z Uras** performed the majority of experiments. Thomas List and Sebastian MB Nijman performed and helped with additional experiments.

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“Your time is limited, so don't waste it living someone else's life.
Don't be trapped by dogma –
which is living with the results of other people's thinking.
Don't let the noise of others' opinions drown out your own inner voice.
And most important, have the courage to follow your heart and intuition.
They somehow already know what you truly want to become.
Everything else is secondary.”

Steve Jobs
1955-2011

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Abstract

How breast cancer patients respond to treatment outcome is not easy to predict and represents a major concern in the application of therapeutics. This study aims to better define personalized treatment options and yield new applications to fighting cancer by two independent approaches.

We first modelled the heterogenous genetic make-up of breast cancer patients and systematically analysed the potential impact of cancer genes on cellular fitness (i.e. cell viability or doubling time) as response to a collection of genetic and chemical perturbations – that is sensitivity or resistance of the cells. We developed a multiplexed screening platform and investigated thousands of potential gene-gene and gene-drug interactions in an isogenic cell line model. Up to 100 genetic alterations could have been interrogated in a single well assay, increasing the number of interactions screened per well so far by up to two orders of magnitude. Considering the clinical relevance and direct implication in breast cancer treatment, we assigned the resistant interaction between PI3K inhibitors and activation of NOTCH and c-MYC as the most interesting. Thus the presented screening strategy can be considered as a powerful tool to gain deeper insight into gene function and drug action, hence to precisely guide clinical treatment decisions.

Oncogenic pathway signatures mirror the biology of cancer and clinical outcome and therefore hold the promise to guide targeted therapy. Given that much attention is being paid on how to specifically and directly attack pathways rather than individual genes, prompted us to search for a therapeutic window targeting the PI3K-PDK1-AKT signalling as this pathway is aberrantly regulated in a large proportion of breast cancer patients and associated with poor prognosis. We zoomed into the PI3K signalling cascade and obtained highly promising insight into the PDK1 regulation.To date, only few mono-ubiquitin conjugated human proteins have been reported yet in all examples this post-translational modification displays a critical regulatory function. Unexpectedly, a diverse panel of human cell lines expressed mono-ubiquitinated PDK1 at varying levels, indicating that this modification is a common and regulated process. The small molecule ubiquitin conjugates to the kinase domain of PDK1 but this attachment does not require PDK1 kinase activity. By applying a library of ubiquitin proteases, we further document the ubiquitin-specific protease 4 (USP4) as a prime enzyme that inhibits PDK1 ubiquitination *in vivo* and *in vitro* and co-localizes

with PDK1 at the plasma membrane, suggesting direct deubiquitination. Together, the regulated modification of PDK1 by a single ubiquitin moiety generates an additional, unpredictable layer of complexity in this critical signalling network. Considering the clinical attractiveness of targeting the ubiquitination machinery, our data provides potential novel therapeutic angles for drug development.

Zusammenfassung

Die Frage wie gut Brustkrebspatienten auf ihre Therapie ansprechen ist sehr schwierig vorherzusagen und daher von enormer Bedeutung. Diese Vorhersage ist jedoch ein Hauptanliegen in der Anwendung neuer Therapeutika. Diese Studie hatte das Ziel, die individuelle Behandlung im Kampf gegen den Krebs besser zu definieren und neue Therapieansätze zu generieren. In dieser Arbeit werden zwei unabhängige Vorgehensweisen erläutert.

Im ersten Teil bedienten wir uns dem heterogenetischen Erscheinungsbild von Brustkrebspatienten. Wir analysierten systematisch den potentiellen Einfluss von diversen Krebs-Genen auf die zelluläre Fitness (z.B. Zellviabilität oder Teilungsrate) als Reaktion auf die Behandlung mit einer Reihe von genetischen oder chemischen Perturbationen. Daraus resultierte Sensitivität oder Resistenz der Zellen. Hierfür entwickelten wir ein multiplexes Screening-Verfahren. Mit Hilfe isogener Tumorzellen konnten wir so tausende potentielle Gen-Gen oder Gen-Pharmaka Interaktionen untersuchen. In einem einzigen Ansatz konnten bis zu 100 verschiedene genetische Varianten gleichzeitig getestet werden. Das erhöhte die Zahl der getesteten Interaktionen in einem Reaktionsansatz auf das Hundertfache. Unter Berücksichtigung der klinischen Relevanz und dem direkten therapeutischen Zusammenhang mit Brustkrebs war die Entdeckung einer resistenten Interaktion zwischen einem Phosphoinositid-3-Kinase (PI3K) Inhibitor und der Aktivierung von NOTCH und c-MYC am interessantesten. Zusammenfassend kann man sagen, dass dieses vorgestellte Screening-Verfahren ein leistungsfähiges Instrument darstellt, um besseren Einblick in Genfunktionen und Wirkung von Pharmaka zu erlangen. Das erleichtert Entscheidungen in der klinischen Anwendung.

Die Signatur von onkogenen Signalwegen spiegelt die Biologie und das klinische Erscheinungsbild von Krebs wieder und ist somit Angriffspunkt spezifisch gerichteter Therapien. Im Moment werden mehr und mehr ganze Signalwege als therapeutischer Angriffspunkt bevorzugt als individuelle Gene dieses Signalweges. Aus diesem Grund suchten wir nach einem möglichen therapeutischen Fenster in der PI3K-PDK1-AKT Signalkaskade. Dieser Signalweg ist bei vielen Brustkrebspatienten aberrant reguliert und führt meist zu einer schlechten Prognose der Patienten. Wir konzentrierten uns auf die PI3K Signalkaskade und erlangten dadurch vielversprechende Einsichten in der Regulation von PDK1. Bis heute

existieren nur wenige Berichte über mono-ubiquitinierte humane Proteine. Aber bei allen Berichten stand diese post-translationale Modifikation in Zusammenhang mit regulatorischen Funktionen. Von uns unerwartet lag das PDK1 Protein mono-ubiquitiniert in verschiedenen humanen Zelllinien vor. Das deutet darauf hin, dass diese Modifikation ein üblicher und regulierter Prozess ist. Die Ubiquitinierung erfolgt in der Kinase-Domäne des PDK1 und ist von der Aktivität der Kinase aber unabhängig. Im Zuge eines Ubiquitin Protease Bibliothek-Screens identifizierten wir die ubiquitin-spezifische Protease 4 (USP4) als wichtigstes und einziges Enzym, welches die Ubiquitinierung von PDK1 *in vivo* und *in vitro* inhibiert und mit PDK1 an der Plasmamembran kolokalisiert. Diese Tatsache deutet auf eine direkte Deubiquitinierung hin. Zusammenfassend fanden wir, dass die regulierte Modifikation des PDK1 durch ein einzelnes Ubiquitin eine zusätzliche und unerwartete Komponente in der Komplexität des zentralen Signalnetzwerkes darstellt. Berücksichtigt man die Attraktivität der Ubiquitin-Maschinerie als neuen therapeutischen Angriffspunkt, so liefern diese neuen Erkenntnisse Potential für die Entwicklung neuer Pharmaka.

Publications arising during this thesis

Smida M[*], Fece de la Cruz F[*], **Uras IZ**, van Jaarsveld R, Muellner MK, Haura EB, Loizou JI & Nijman SM. Ataxia Telangiectasia Mutated (ATM) mutations in lung cancer are synthetic lethal with MEK inhibitors. Nature 2013 (submission) ([*] equal contribution)

Muellner MK[*], Duernberger G[*], Ganglberger F[*], Kerzendorfer C, **Uras IZ**, Schonegger A, Bagienski K, Colinge J & Nijman SM. TOPS: A versatile software tool for statistical analysis and visualization of combinatorial gene-gene and gene-drug interaction screens. BMC Bioinformatics 2013 (submitted) ([*] equal contribution)

Uras IZ, List T & Nijman SM. Ubiquitin-specific protease 4 inhibits mono-ubiquitination of the master growth factor signaling kinase PDK1. PLOS ONE. 2012;7(2):e31003. doi: 10.1371/journal.pone.0031003.

Muellner MK, **Uras IZ**[*], Gapp BV[*], Kerzendorfer C, Smida M, Lechtermann H, Craig-Mueller N, Colinge J, Duernberger G & Nijman SM. A chemical-genetic screen reveals a mechanism of resistance to PI3K inhibitors in cancer. Nat Chem Biol. 2011 Sep 25;7(11):787-93. doi: 10.1038/nchembio.695. ([*] equal contribution)

Perne A[*], Muellner MK[*], Steinrueck M, Craig-Mueller N, Mayerhofer J, Schwarzinger I, Sloane M, **Uras IZ**, Hoermann G, Nijman SM & Mayerhofer M. Cardiac glycosides induce cell death in human cells by inhibiting general protein synthesis. PLOS ONE. 2009 Dec 16;4(12):e8292. doi:10.1371/journal.pone.0008292. ([*] equal contribution)

Abbreviations

17AAG	17-allylamino-17-demethoxygeldanamycin
4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1
53BP1	p53 binding protein 1
A residue	Alanine residue
ABC	ATP binding cassette
AC	Doxorubicin, cyclophoshamide
AGC	cAMP-dependent, cGMP-dependent, protein kinase C
AML	Acute myeloid leukemia
AT	Doxorubicin, docetaxel
ATM	Ataxia telangiectasia mutated
ATP	Adenosine-5'-triphosphate
ATR	Ataxia telangiectasia and Rad3-related protein
BAD	BCL2 antagonist of cell death
BAP1	BRCA1 associated protein 1
BCC	Basal cell carcinoma
BCL2	B cell lymphoma 2
BCR-ABL	Break point cluster-Abelson
BECN1	Beclin 1
BIM	Bcl-2-like protein 11 (apoptosis facilitator)
BMI	Body mass index
BMP	Bone morphogenic protein
bp	Base pair
BRCA1	Breast cancer 1, early onset
BRCA2	Breast cancer 2, early onset
BRCC	BRCA1 and BRCA2 containing complex
BRCC36	BRCA1/ BRCA2 containing complex subunit 3
BSA	Bovine serum albumin
C residue	Cysteine residue
c-kit	Mast/stem cell growth factor receptor (SCFR); CD117
c-MYC	V-myc myelocytomatosis viral oncogene homologue (avian)
C. elegans	Caenorhabditis elegans
CADM1; IGSF4	Cell adhesion molecule 1
CAF status	Status of coronary artery fistula
CAF/FAC	Cyclophosphamide, doxorubicin, fluorouracil (5-FU)
CCNE1	Cyclin E1
CDC2	Cell division control protein 2
CDC73	Cell division cycle 73
CDKN1	Cyclin-dependent kinase inhibitor 1
CDKN1B	Cyclin-dependent kinase inhibitor 1B; p27
CDKN1C	Cyclin-dependent kinase inhibitor 1C; p57

CDKN2A	Cyclin-dependent kinase inhibitor 2A
CDKN2A	p16-INK4A
CDKN2A	p14-ARF (alternate open reading frame)
cDNA	Complementary DNA
CHEK2	Serine/threonine-protein kinase Chk2
CHG	Copy number
CMF	Cyclophosphamide, methotrexate, fluorouricil
CRM1	Chromosome region maintenance 1
CSF-1R	Colony stimulating factor 1 receptor
CT-antigen	Cancer-testis antigen
CTC features	Circulating tumour cell features
ctrl	Control
CYLD	Cylindromatosis gene (turban tumour syndrome)
Cyt	Cytoplasmic
DAPI	4,6-diamidino-2-phenylindole
DAPK1	Death-associated protein kinase 1
dH_2O	Distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
Downreg	Downregulated
DTT	Dithiothreitol
DUB	Deubiquitinating enzyme; deubiquitinase
DV	Destination vector
E residue	Glutamic acid residue
EC	Epirubicin, docetaxel
EC vector	Entry clone vector
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eIF4	Eukaryotic initiation factor 4
eIF4E	Eukaryotic translation initiation factor 4E
ER	Oestrogen receptor
ER degradation	Endoplasmatic reticulum degradation
ERBB	Erythroblastic leukemia viral oncogene homologue
ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homologue 2; neuro/glioblastoma derived oncogene homologue (avian)
Erk	Extracellular signal-regulated kinase
ESCRT	Endosomal sorting complexes required for transport
ETS	E-twenty six
F1 ori	F1 phage origin
FACS	Fluorescence activated cell sorting

FANCD2	Fanconi anemia complementation group D2
Fas-L	Fas ligand
FASLG	Fas antigen ligand
FAT2	FAT tumour suppressor homologue 2
FAT3	FAT tumour suppressor homologue 3
FDA	United States (US) Food and Drug Administration
FDR	False discovery rate
FEC	Fluorouracil, epirubicin, cyclophosphamide
FGFR	Fibroblast growth factor receptor
FH	Fumarate hydratase
FHIT	Fragile histidine triad protein
FLCN	Folliculin
Flt4	Fms-related tyrosine kinase 4
Fos	Finkel-Biskis-Jinkins (FBJ) murine osteosarcoma viral oncogene homologue
FoxO	Forkhead box O
FoxO-3a	Forkhead box O3
FOXP3	Forkhead box P3
Fwd	Forward
FWF	Austrian Science Fund (Fonds zur Förderung der wissenschaftlichen Forschung)
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	Genomic DNA
GFP	Green fluorescing protein
GFR	Growth factor receptor
Gly	Glycine
GSI	Gamma secretase inhibitor
GSK3	Glycogen synthase kinase-3
GT	Gemcitabine, taxol
H1	Histone H1
HA	Haemaglutinin (tag)
HBS	HEPES buffered saline
HCF-1	Host cell factor 1
HDAC	Histone deacetylase
HDM2	Human MDM2
HECT	Homologous to the E6AP carboxyl terminus
HEK293T	Human embryonic kidney 293 cells transformed by expression of the large T antigen from SV40
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER1	Human epidermal growth factor receptor 1
HER2	Human epidermal growth factor receptor 2
HIF-1α	Hypoxia-inducible factor 1α
His	Histidine (tag)
HLAMP	High-level amplification
HNSCC	Head and neck squamous cell carcinoma
HOMD	Homozygous deletion

HPV	Human papilloma virus
HR	Hormonal receptor
HSP90	Heat shock protein 90
Hygro	Hygromycin
i.e.	Id est
ICN1	Intracellular active domain of NOTCH1
IDC NOS	Invasive ductal carcinoma not otherwise specified
IGF-1	Insulin-like growth factor-1
IGF1-R	Insulin-like growth factor 1 receptor
IKBKE	Inhibitor of nuclear factor kappa light polypeptide gene enhancer in B cells, kinase epsilon
IKK	Inhibitor of kappa B (IκB) kinase
ILC	Invasive lobular carcinoma
INPP4B	Type II inositol 3,4-bisphosphate 4-phosphatase
IP	Immunoprecipitation
IRES	Ribosome entry site
ISG15	Interferon stimulated gene (ISG) 15
IκB	Nuclear factor kappa light polypeptide enhancer in B cells inhibitor
JAG1	Jagged 1 protein
JAK2	Janus kinase 2
JAMM/MPN	JAMM motif proteases; Jab1/Mov34/Mpr1 Pad1 N-terminal+ (MPN+)
Jun	V-jun avian sarcoma virus 17 oncogene homologue
K-less	Lysine-less
KD	Kinase domain
kDa	Kilodalton
KLF6	Krüppel like factor 6
KRas	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue
LB medium	Lysogeny broth
LTR	Long term repeat
Lys; K residue	Lysine residue
MAPK	Mitogen-activated protein kinase
Mb	Megabase
MDM2	Mouse double minute 2 homologue
Mek	MAPK/extracellular signal-regulated kinase kinase
Mem	Membrane bound
MEN1	Multiple endocrine neoplasia 1
Met	Mesenchymal-epithelial transition factor (MET) receptor tyrosine kinase
miRNA	microRNA
MJD	Machado-Joseph disease protein domain protease
MMC	Mitomycin C
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
mTORC	mTOR complex
Mut	Mutated
N	Node status

NA	Not available
NAE	E1-activating enzyme for neddylation
NBN	Nibrin; NBS1
NCCN	National Comprehensive Cancer Network
NCI	National Cancer Institute
ND	Not determined
NEDD4	Neural precursor cell expressed developmentally downregulated protein 4
Neo	Neomycin
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NF1	Neurofibromin 1
NF2	Neurofibromin 2
NIH 3T3	3-day transfer, inoculum 3 x 10^5 cells
NOTCH-delta E	NOTCH1 mutant lacking the extracellular domain
NOTCH1	Notch homologue 1, translocation-associated (Drosophila)
NP-40	Nonylphenoxylpolyethoxyethanol
Nuc	Nuclear
NUMB	Numb homologue (Drosophila)
OTU	Ovarian tumour protease
OTUB1	Ovarian tumour (OTU) domain, ubiquitin aldehyde binding 1
OX40	Tumour necrosis factor receptor superfamily, member4; TNFRSF4
PALB2	Partner and localizer of BRCA2
PARP	Poly-adenosine diphosphate (ADP)-ribose polymerase
PARP1	Poly (ADP-ribose) polymerase 1
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PDGFR-α	Platelet-derived growth factor alpha receptor
PDK1	3-phosphoinositide-dependent protein kinase 1; phosphoinositide-dependent kinase 1
PGK	Phosphoglycerate kinase
PH domain	Pleckstrin Homology domain
pH value	Potential (of) hydrogen
PI3K	Phosphatidylinositol 3-kinase; phosphoinositide 3-kinase
PIF	PDK1-interacting fragment
PIK3CA	PI3K catalytic subunit α-isoform
PIK3CB	PI3K catalytic subunit β-isoform
PIK3R1	PI3K regulatory subunit-α
PIP2	Phosphatidylinositol (4,5)-biphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PLK1	Polo-like kinase 1
PLpro	SARS coronavirus DUB papain like protease
PPP2R1B	Protein phosphatase 2 subunit A (PR65)

PR	Progesterone receptor
PRAS40	Proline-rich AKT substrate, 40 kDa; proline-rich AKT1 substrate 1
PTCH1	Patched homologue 1
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
Puro	Puromycin
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative real-time PCR
R residue	Arginine residue
RAC1	Ras-related C3 botulinum toxin substrate 1
RAF	Rapidly accelerated fibrosarcoma
RAS	Rat sarcoma
RASV12	A mutated form of Ras with a glycine-to-valine mutation at residue 12
RB	Retinoblastoma
RB1	Retinoblastoma protein 1
Res	Resistant
Rev	Reverse
RING domain	Really interesting new gene domain
RIP1	Receptor-interacting protein 1
RIPA buffer	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RNAi	RNA interference
Robo1	Roundabout 1
RPMI-1640	Roswell Park Memorial Institute medium
RPS6	40S ribosomal protein S6
RSK	90 kDa ribosomal protein S6 kinase; p90 ribosomal S6 kinase
RTK	Receptor tyrosine kinase
S. cerevisiae	Saccharomyces cerevisiae
S. pombe	Schizosaccharomyces pombe
S.d.	Standard deviation
S6K	Ribosomal S6 kinase; p70 S6 kinase
SAHA	Suberoylanilide hydroxamic acid
SAPE	Streptavidin-coupled phycoerythrin
SCF complex	Skp, Cullin, F-box containing complex
SDHD	Succinate dehydrogenase subunit D
SDS	Sodium dodecyl sulphate
Sen	Sensitive
Ser; S residue	Serine residue
SERM	Selective oestrogen receptor modulator
SGK	Serum- and glucocorticoid-induced kinase
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
Skp2	S-phase kinase-associated protein 2
SMAD2	SMAD family member 2
SMAD4	SMAD family member 4

SNP	Single nucleotide polymorphism
Src	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homologue (avian)
STAT3	Signal transducer and activator of transcription 3
STK11	Serine/threonine kinase 11; LKB1
Strep	Streptavidin
T	Tumour size
TAK1	Mitogen-activated protein kinase kinase kinase 7; MAP3K7
TAP	Tandem affinity purification
TBC	tre-2/USP6, BUB2, cdc16
TC	Docetaxel, capecitabine
TCF	T cell factor
TE	Tris-EDTA
TGF-β	Transforming growth factor beta
Thr; T residue	Threonine residue
Tie-2	TEK tyrosine kinase, endothelial
TNBC	Triple-negative breast cancer
TP53	Tumour suppressor 53; tumour protein p53
TRAF6	Tumour necrosis factor (TNF) receptor associated factor 6
TSC1	Tuberous sclerosis 1
TSC2	Tuberous sclerosis 2
TWIST1	Twist-related protein 1
Ub	Ubiquitin
Ub-AMC	Ubiquitin-7-amino-4-methylcoumarin
Ub-PDK1	Ubiquitinated PDK1
UCH	Ubiquitin C-terminal hydrolase
Upreg	Upregulated
USP	Ubiquitin-specific protease
USP4	Ubiquitin-specific protease 4
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VHL	Von Hippel-Lindau tumour suppressor
vs.	Versus
WB	Western blot
WIP1	Wild type p53-induced phosphatase
WNT	Wingless-type mouse mammary tumour virus (MMTV) integration site family
WT	Wild type
WT1	Wilms tumour 1
WTX	Wilms tumour X-associated
WWTF	Vienna Science and Technology Fund
z.B.	Zum Beispiel

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1 Introduction

1.1 Breast cancer

The last decades have painfully taught us that fighting cancer resembles the battle against the “Father of the deadly monsters“ of the ancient Greek mythology -the dragon Typhon- who with his 100 heads was nearly impossible to defeat even by Zeus -the “Father of Gods and men”- (Majewski & Bernards, 2011). In fact, the global burden of cancer increases incredibly fast due to the aging and growth of world population. Another relentless contributor is the adoption of cancer-associated lifestyle choices that are in particular smoking, physical inactivity and westernized diets in developing countries (Jemal et al, 2011). Henceforth, by 2030, cancer deaths in the world are projected to continue rising to over 13 million (Ferlay et al, 2010).

Cancer encompasses a collection of more than 100 diseases with distinct genetic make-up, various risk factors and epidemiology (Stratton et al, 2009). Regardless of sex, among the five major cancer sites are lung, colorectum, breast, prostate and pancreas (Siegel et al, 2012a; Siegel et al, 2012b). Among women in western society, breast cancer is the most common leading cancer type and remains a disease with high incidence, mortality and morbidity (Ocana & Pandiella, 2008). The most sobering reality is that one in eight women will develop breast cancer in her lifetime. The global incidence is annually increasing and has been projected to be over one million in 2008, making breast cancer the most frequently diagnosed malignancy (Figure 1) (Clarke et al, 1990; Jemal et al, 2011; Lott et al, 2009; Siegel et al, 2012b). Though much less common than in women, it also affects men, occurrence being just 1% of that in women. Although it is largely associated with middle and old age, breast cancer also occurs during younger ages. This group displays a more aggressive disease and poor prognosis. Indeed, at younger ages, breast cancers often progress to highly malignant tumours with invasive and metastatic properties leading to both decreased overall and disease-free survival (Lott et al, 2009; Zhou & Recht, 2004).

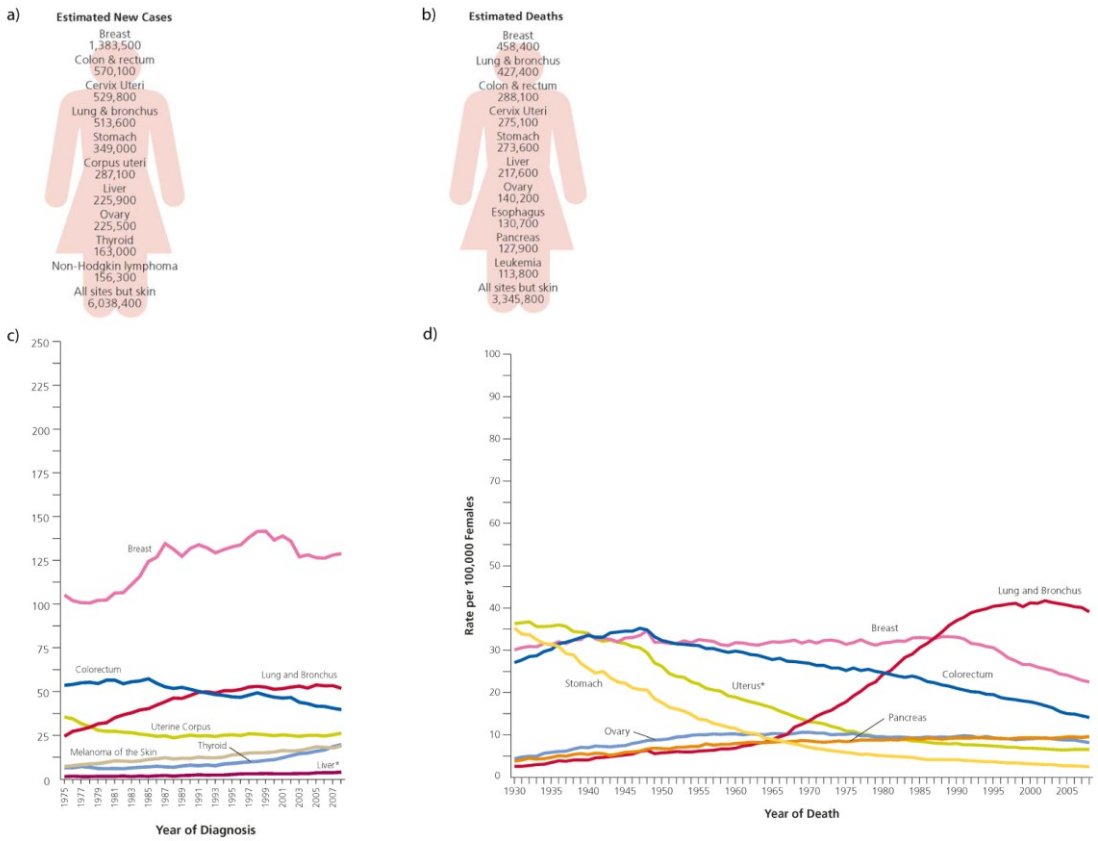

Figure 1. Trends in incidence and mortality rates for leading cancers among women

Adapted from (Jemal et al, 2011) (a-b) and (Siegel et al, 2012b) (c-d). a) Estimated new cancer cases in women: Breast cancer is the most frequently diagnosed malignancy worldwide. b) Estimated deaths in women: Breast cancer is the leading cause of cancer death worldwide. c) Diagnosis rates for selected cancers among females in the United States, 1975 to 2008: Breast cancer shows the highest incidence rate. Rates are age adjusted to the 2000 US standard population and adjusted for delays in reporting. d) Death rates among females for selected cancers in the United States, 1930 to 2008: After lung and bronchus cancer, breast cancer shows the highest death rate. Rates are age adjusted to the 2000 US standard population. (a-b) **Confirmation Number: 11107151 Citation Information Order Detail ID:** 63848958 **CA by AMERICAN CANCER SOCIETY Reproduced with permission of LIPPINCOTT WILLIAMS & WILKINS in the format reuse in a dissertation/thesis via Copyright Clearance Center.** (c-d) **Confirmation Number: 11107151 Citation Information Order Detail ID:** 63848962 **CA by AMERICAN CANCER SOCIETY Reproduced with permission of LIPPINCOTT WILLIAMS & WILKINS in the format reuse in a dissertation/thesis via Copyright Clearance Center.**

Breast cancer is not a single disease but encompasses multiple subgroups. Each of them harbours a unique gene expression profile and associated clinical outcome (Figure 2) (2012; Campbell & Polyak, 2007; Sorlie et al, 2001). The origin is widely thought to be a single cell with multiple mutations yet still little is known about how breast cancer evolves (Campbell & Polyak, 2007; Fialkow, 1979; Hahn & Weinberg, 2002; Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011).

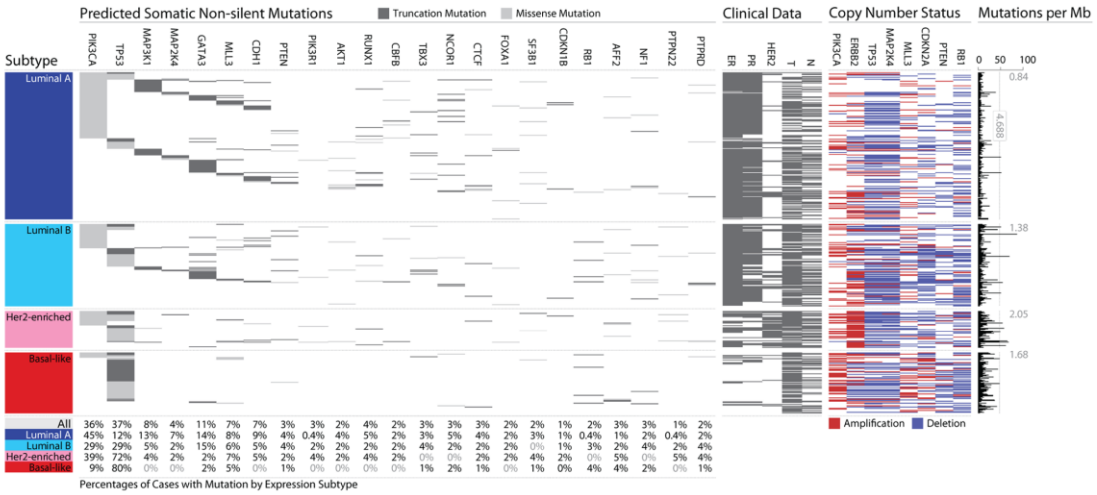

Figure 2. Somatic mutation spectrum in human breast cancer and correlations with genomic and clinical characteristics

Adapted from (2012). Breast tumour samples are clustered based on mRNA-expression subtype: luminal A (n=225), luminal B (n=126), HER2-enriched (n=57) and basal-like (n=93). The left panel illustrates non-silent somatic mutation spectrum and frequencies for significantly mutated genes. The middle panel shows clinical patterns: dark grey, positive or T2-4; white, negative or T1; light grey, N/A or equivocal. N, node status; T, tumour size. The right panel shows the copy number status of significantly mutated genes. Red is indicative for frequent copy number amplifications. Blue means deletions. The far-right panel displays non-silent mutation rate per tumour (mutations per megabase, adjusted for coverage). The average mutation rate for each mRNA-expression subtype is given. Hypermutated means mutation rates >3 s.d. above the mean (>4.688, indicated by grey line).

Breast cancer is composed of a mixed population of cells including normal cells, stroma and infiltrating leukocytes (Bertos & Park, 2011). The vast majority of breast tumours are structurally categorized into ductal and lobular subtypes, the former being the most frequent (Bertos & Park, 2011; Li et al, 2005). The remainder are referred to as tubular, medullary, apocrine, neuroendocrine, metaplastic, comedo, mucinous A and B, inflammatory, adenoid cystic and micropapillary (Bertos & Park, 2011; Li et al, 2005; Weigelt et al, 2010). Importantly, histological types are associated with clinical outcome (Arpino et al, 2002; Diab et al, 1999; Pedersen et al, 1995; Rakha et al, 2010; Weigelt et al, 2010). The capability to invade into other tissues defines breast tumours as in situ, invasive and metastatic (Campbell & Polyak, 2007; Simpson et al, 2005). Consequently, tumour development is pinpointed by histo-pathological stages with a broad spectrum of hyperplasia of mammary duct epithelial cells, ductal carcinoma in situ alongside invasive tumour

formation, lymph node involvement and metastases to distant organs of the body (Kadota et al, 2009). The prognostic predictions of early disease are primarily determined by the main clinical parameters such as age, histological tumour grade, tumour size and number, lymph node status and presence of distant metastases (Singletary et al, 2003; Singletary & Connolly, 2006; Singletary & Greene, 2003).

Significant advances in diagnosis and therapy have been achieved in the last decades. Yet, we still have to face several unresolved clinical and scientific problems. These are related to the facts that a) rates of incidence and recurrence remain high, b) despite some success stories, therapy is usually not individualized, can lead to severe side effects and is often not curative (Campbell and Polyak, 2007). Thus, more therapy options are sorely needed. We need to know how to prevent the disease and how to develop more specific and sensitive diagnostic methods. Resolving all these problems represents a big challenge largely because the precise biological mechanisms underlying breast cancer initiation and progression as well as those leading to treatment resistance remain still poorly understood (Campbell & Polyak, 2007; Polyak, 2007). Importantly, not every cancer-driving event is druggable. As a consequence, the translation of our knowledge into therapies remains challenging.

1.1.1 Breast cancer taxonomy

Before stepping on the battlefield to finally win the war on this deadly beast called breast cancer, we have to understand the followings: the uniqueness of each tumour, its interaction with micro- and macro-environment, tumour molecular classification and the striking nature of molecular heterogeneity between each tumour and within a single tumour. Only after a better understanding of these points, we can move forward to improved personalized medicine.

1.1.1.1 Breast cancer evolution

In 1976, Peter Nowell said: “More research should be directed towards understanding and controlling the evolutionary process in tumours before it reaches the late stage seen in clinical cancer” (Nowell, 1976). More than three decades have passed and still the evolution of breast cancer is not easy to predict.

Just as all other types of cancer, breast cancer is driven by somatically acquired point mutations and chromosomal rearrangements. In the classical view, mutations occur usually random and as independent events that can be accelerated by exogenous carcinogens or DNA repair defects. After clonal selection through Darwinian competition, those somatic mutations promoting a selective growth advantage on the cell drive successive waves of expansion. The fittest clone dominates the cellular compartment and leads to cancer. Thus, the conventional model of tumour progression is one of gradualism in which mutations occur cumulatively over time throughout the lifespan of an individual (Greaves & Maley, 2012; Nowell, 1976).

In contrast to this gradual model, however, evidence for a punctuated clonal evolution has been recently provided by single-nucleus sequencing of two breast cancer cases (Navin et al, 2011). Accordingly, analysis of 100 single cells from the same tumour sample revealed that tumour subpopulations are each distant from their root with few persistent intermediates. Importantly, this observation mirrors the sudden emergence of a tumour cell in which the effective population growth rate exceeds the rate of genomic evolution.

Moreover, as opposed to ordered mutational processes, improved mathematical models revealed predictions about convergent and divergent evolution within the same tumour (Beckman et al, 2012). Hence, heterogeneous genetic and epigenetic changes within a single tumour lead to completely different sets of signalling interactions to achieve common ultimate tumour phenotypes, reflecting convergent evolution (Pai et al, 2009). These modelled predictions have been verified not only in renal cell cancer whereby different genetic alterations affecting the same pathway were separated within the primary tumour (Gerlinger et al, 2012) but also reflected in breast cancer samples manifesting genetic differences observed between the primary tumours and metastases (Pai et al, 2009; Shah et al, 2009). Regrettably, this convergent nature of breast cancer presents numerous prevention and treatment challenges.

In addition, next-generation sequencing analysis described a phenomenon termed chromothripsis -from the Greek for shattered chromosomes- (Stephens et al, 2011). In this process, tens to hundreds of genomic rearrangements occur in a single cellular catastrophe. Explicitly, one or two whole chromosomes are shattered into pieces and stuck back together randomly, creating frequent fluctuations between two

copy number states. These states are thought to be highly unlikely if such genomic disruptions occur gradually and thus indicate a one-off cellular crisis. More than one cancer-causing lesion can emerge out of such a single chaotic crisis that can inactivate transcription of many more genes. The acquired selective advantage at hypermutable genomic loci is then expected to promote evolution toward cancer.

Furthermore, based on the age-incidence statistics, mathematical modelling of tumour development has indicated the requirement of five to eight rate-limiting events (Armitage & Doll, 1954; Stratton et al, 2009). This model was further supported by studies in which normal primary human cells can be fully converted into cancer cells when functional changes are engineered in at least five to six genes (Schinzel & Hahn, 2008). Intriguingly, studies on various genetic and epigenetic features in samples from different tissues have previously demonstrated that the regional mutation rates in cancer genomes are strongly influenced by chromatin organization (Schuster-Bockler & Lehner, 2012). Explicitly, mutation rates are increased in more heterochromatin-like domains and repressed in more open chromatin. This is thought to mirror variations in accessibility to DNA repair complexes, in signal repair ability or elevated mutagen exposure at the nuclear periphery.

Our knowledge on the dynamics of breast cancer development is further expanding. Recent bioinformatics algorithms on 21 primary breast tumours reconstructed the genomic life history of breast cancer and suggested that the total breast tumour mass is composed of a mixture of multiple discrete subclones (Nik-Zainal et al, 2012b). Yet, every tumour harbours one dominant subclonal lineage and its expansion to more than 50% of tumour cells may be the final rate-limiting step in breast cancer development triggering diagnosis (Figure 3) (Nik-Zainal et al, 2012b). In addition, mathematical analysis of the complete genome sequence of these 21 tumours was applied to a better understanding of their mutational signatures with a particular focus on DNA damage and repair pathways (Nik-Zainal et al, 2012a). Here, five biologically distinct classes of base substitutions were identified occurring either simultaneously or at different evolutionary stages. The presence of regional hypermutation clusters termed as kataegis -from the Greek for shower or thunderstorm- was observed in more than half of the breast tumours analysed. Regions of kataegis were different in each tumour. Many co-localized with somatic rearrangements and some displayed features of chromothripsis. Base substitutions in these regions were mainly clusters of cytosine at TpC dinucleotides. Intriguingly,

kataegis was not seen in previously published mutation catalogues of melanoma and lung cancer (Nik-Zainal et al, 2012a; Pleasance et al, 2010a; Pleasance et al, 2010b). Altogether, these whole genome sequence studies have started to unfold and better characterize mutational processes in breast cancer at a level that was not possible before.

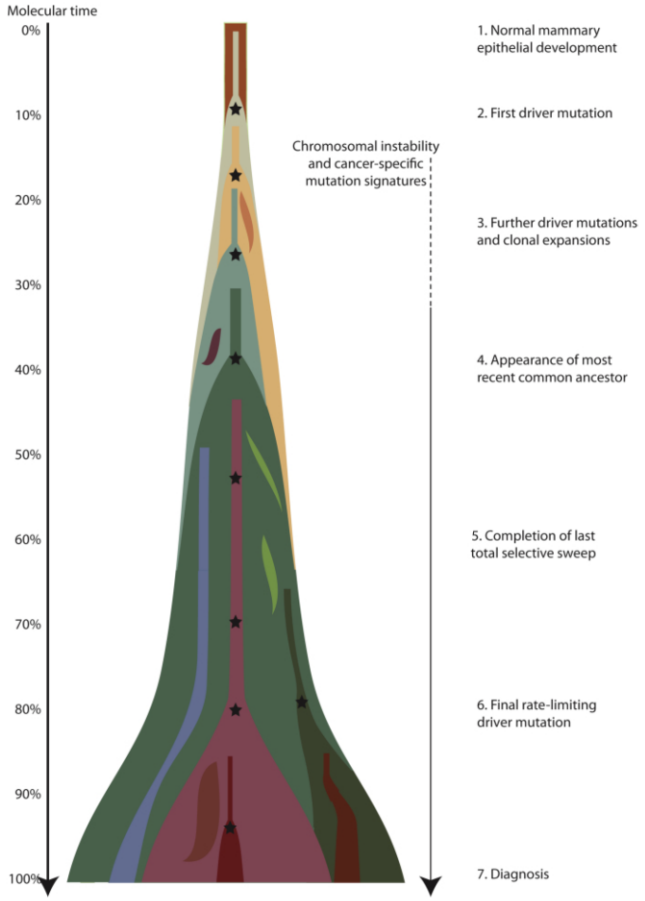

Figure 3. A model for dynamics of breast cancer evolution over molecular time

Adapted from (Nik-Zainal et al, 2012b). A cancer develops through acquisition of driver mutations which yield clonal expansions. The driver mutations arise only sporadically in long-lived lineages of cells which passively acquire several diverse alterations without expansion. Driver mutations are indicated with black stars.

1.1.1.2 Repertoire of somatic mutations and affected breast cancer genes

A pressing question in breast cancer research is: What does the catalogue of somatic mutations along with the cancer genes affected in breast cancer look like?

By the time of diagnosis, each breast tumour has acquired thousands to hundreds of thousands somatic molecular changes driving uncontrolled cellular growth and indefinite proliferation (Stratton, 2011; Wong et al, 2011). Genomic modifications underlying cancer are any changes in the sequence of the genome including point mutations affecting single base pairs, deletions or insertions, loss of heterozygosity, alternative splicing, amplifications or translocations as well as epigenetic deregulation (Figure 4) (Ha et al, 2012; Haber & Settleman, 2007; Vogelstein & Kinzler, 2004). The concept of epigenetic silencing encompasses heritable aberrations of nucleotides and histones. Single base changes modify protein activity. Deletions, insertions and amplifications alter the abundance of a gene and its product, whereas alternative splicing and translocations yield novel proteins (Dancey et al, 2012).

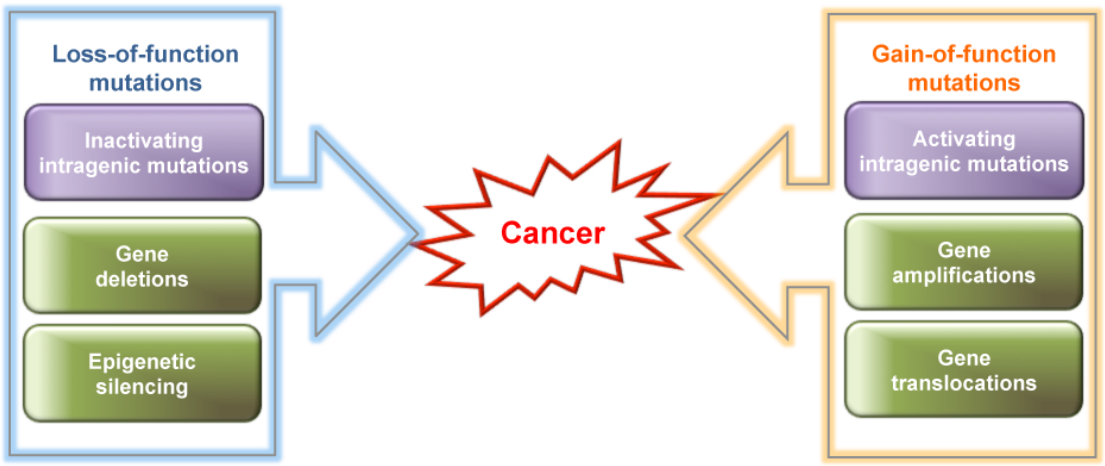

Figure 4. Spectrum of molecular alterations steering human cancer

A cancer cell may transfer different modifications to its progeny: The gain-of-function mutations activate certain genes. The loss-of-function mutations cause inactivation of distinct genes. Intragenic mutations that are small nucleotide changes within genes (highlighted in purple) present drivers of a mutational event underlying human cancer. Heritable alterations of nucleotides and histones in regulatory regions of genes are implicated in epigenetic silencing which represses gene expression when a certain mutation is missing. Different chromosomal fragments can be rearranged upon translocations yielding either a novel, aberrant fusion protein or an abnormal expression of a normal gene.

Molecular alterations are categorized into drivers and passengers. Driver mutations as the name already whistle-blows drive tumourigenesis. They confer a selective growth advantage to a cell thus enhance tumour evolution and are positively selected. By definition, driver mutations occur in cancer genes. It is remarkable that more than 350 (≥1.6%) of the approximately 22.000 protein-coding genes in the human genome have been reported as cancer genes by various physical and genetic

mapping strategies, biological assays and as plausible biological candidates (Futreal et al, 2004; Greenman et al, 2007; Stratton et al, 2009). Yet, the true number of cancer genes still remains unknown. In contrast, the majority of mutations is passengers that are thought to be rather indicative for high mutation rate resulting from carcinogens and DNA instability (Dancey et al, 2012). Passengers likely appear to have little to no functional consequences in cancer progression. Nevertheless, it is important to point out that passengers show great diversity in the number and interactions between passenger alterations, many different signalling pathways and biological processes (Ogino et al, 2012). Indeed, accumulating evidence indicates that even though each passenger alone may exert a very small effect on tumour properties, a net effect of all small effects of all the passengers may be substantial enough (Ogino et al, 2012). Strikingly, the fact that some of these passenger mutations affect large genomic regions harbouring many genes brings up the persisting question: Can such large genomic disruptions be entirely passengers? Recent studies indicate that the cumulative response of genes affected by such a class of mutation called hemizygous focal deletion is sufficient to optimize proliferative capability in tumourigenesis (Solimini et al, 2012).

Cancer genes responsible for the neoplastic process of breast cancer development and progression fall into three classes: oncogenes, tumour suppressor genes and stability genes (Vogelstein & Kinzler, 2004). Importantly, combinations of mutations in these three classes of genes can cause breast cancer by aberrant regulation of proliferation, differentiation, apoptosis, tissue architecture and angiogenesis (Fish & Molitoris, 1994; Muthuswamy, 2009; Vogelstein & Kinzler, 2004).

Oncogenes are targeted by alterations so that the gene gets constitutively active or active only under conditions in which the wild type gene is not. Such aberrations include copy number alterations increasing gene dosage, single nucleotide mutations, chromosomal rearrangements along with epigenetic modifications (Brown & Botstein, 1999; Davies et al, 2002; Feinberg & Tycko, 2004; Jones & Baylin, 2002; Polyak & Riggins, 2001; Vogelstein & Kinzler, 2004). Around 90% of the known cancer genes with somatic mutations act dominantly, that is, a selective proliferative advantage is bestowed by an activating somatic mutation in just one allele of an oncogene (Davies et al, 2002; Stratton et al, 2009).

How a tumour suppressor gene is targeted by alterations is exactly the opposite. Here, the activity of the gene product is reduced by mutations. Thus, such

inactivating mutations switch off the suppressive action of a tumour suppressor gene promoting tumour growth. In his two-hit hypothesis, Knudson proposed that in order to promote tumourigenesis both alleles of the gene need to receive inactivating mutations, suggesting a recessive act (Knudson, 2002). Such inactivating mutations arise from missense mutations at functionally important residues, from nonsense mutations resulting in a truncated protein, from large-scale deletions of entire genes from the chromosome, along with epigenetic silencing leading to malfunction or absence of the encoded protein (Greenman, 2012). In some cases, loss of only one allele of a gene results in reduction in functional competence and proves beneficial during tumourigenesis (Santarosa & Ashworth, 2004). These so-called hemizygous deletions target a large number of haploinsufficient tumour suppressor genes and simultaneously avoid deletion of essential ones (Greenman, 2012; Solimini et al, 2012).

In addition, a third class of breast cancer genes is known as stability genes and keeps the genetic alterations to a minimum (Vogelstein & Kinzler, 2004). This class includes nucleotide-excision repair, base-excision repair and mismatch repair genes. Others function as caretakers of processes involving large portions of chromosomes such as those responsible for mitotic recombination and chromosomal segregation like *BRCA1*. In contrast to oncogenes and tumour suppressor genes, the inactivated stability genes increase the likelihood of mutations in other genes. Consequently, all genes may be affected by mutated stability genes but only oncogene and tumour suppressor aberrations prove beneficial for a selective growth advantage. Similar to tumour suppressor genes, both alleles of stability genes need to be inactivated for a physiological effect to arise. Although the molecular underpinnings of aneuploidy and chromosome instability are still largely unknown, some candidate genes and pathways have been revealed. These cover cell cycle checkpoints, telomere crisis or centrosome duplication (Curtis et al, 2012; Daniel et al, 2011; Maser & DePinho, 2002; Pihan & Doxsey, 2003; Rajagopalan et al, 2003).

Mutations in the cancer genes categorized into three groups as described above can occur in the germline predisposition leading to hereditary breast cancers or in single somatic cells resulting in the formation of sporadic tumours (Vogelstein & Kinzler, 2004). In fact, around 10% of breast cancer patients exhibit a hereditary predisposition, mainly caused by mutations in *BRCA1* and *BRCA2* as well as *TP53* and *PALB2* (Catucci et al, 2012; Polyak, 2007; Poumpouridou & Kroupis, 2012; Pylkas et al, 2012; Rouleau et al, 2012; Vogelstein & Kinzler, 2004). The majority,

however, arises sporadically from somatic mutations or chromosome instabilities in the breast tissue (Kadota et al, 2009).

More than 10 years ago at the turn of the millennium, reference human genome sequence promised novel strategies and opportunities for surveying cancer genomes. In fact, the rapid development of new genomic technologies including DNA microarray analysis, next-generation sequencing, comparative genomic hybridization technologies for copy number profiling along with tissue microarrays and reverse-phase protein lysate arrays have remarkably expanded our understanding of the complex landscape of cancer genes and mutational processes. Surely, this is nowhere more obvious than in breast cancer. Indeed, 70-gene gene signature profiles for breast cancer samples enabled us to distinguish between good and bad prognosis (van 't Veer et al, 2002). Genome-association studies identified novel breast cancer susceptibility loci along with new single nucleotide polymorphisms (Turnbull et al, 2010). Analyses of somatic mutations and copy number variants discovered numerous molecular aberrations in several new cancer genes, each of them potentially pinpointing a novel target for therapeutic intervention (Banerji et al, 2012; Curtis et al, 2012; Polyak, 2007; Shah et al, 2012; Stephens et al, 2012).

Importantly, we have now learnt to appreciate that the number of genes mutated in breast cancer patients is fairly high (Figure 5) (Stephens et al, 2012). Surely, all these studies do not represent the whole picture but only emphasize the sobering notion that driver mutations and mutational processes operative in breast cancer have not yet been comprehensively explored. Nevertheless, these newly identified genes that drive breast cancer could change the way of diagnosis and create novel avenues for next-generation treatments.

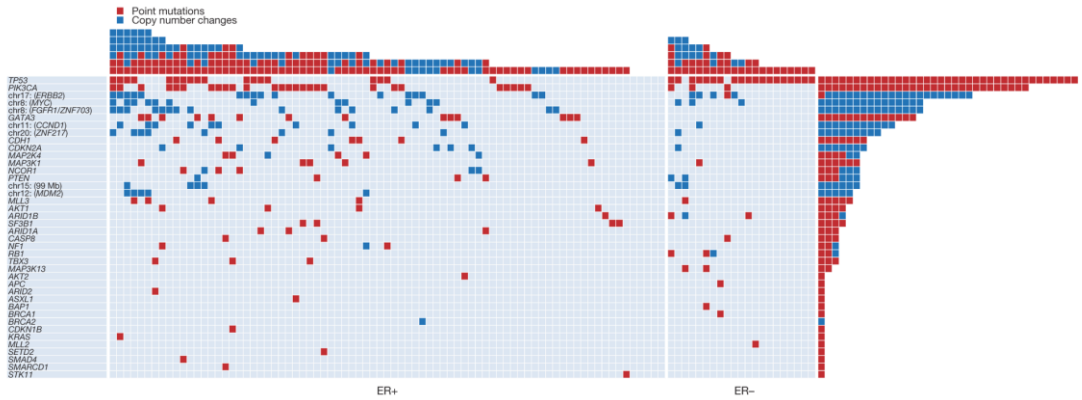

Figure 5. The landscape of breast cancer-driving mutations

Adapted from (Stephens et al, 2012). The far-left panel lists all 40 genes with a driver mutation or copy number change identified. Rows display the number of mutations in each gene in the 100 tumours studied. Columns show the number of driver mutations in each breast cancer. The colour code red and blue indicates point mutations and copy number changes, respectively.

However, despite few exceptions, we must be aware of the fact that the frequency of mutations in any gene in sporadic tumours is low (Polyak, 2007; Stephens et al, 2012). Hence, the discovery of all mutated genes will require the sequencing of large sets of tumours representing each breast cancer subtype in diverse ethnic populations. In addition, there is a dire need to address the functional relevance of these mutations and to understand which ones provide a growth or survival advantage for the cancerous cell.

Surely, the low frequency of mutations seems daunting for the development of targeted therapies but of great importance, studies also linked the molecular profiles of breast cancer subtypes with known signalling pathways (Curtis et al, 2012). Strikingly, mutations and affected genes are clustered in a limited number of pathways. These include PIK3CA/AKT/PTEN, IKBKE/NF-κB, p53-related pathways, chromatin remodelling, ERBB signalling as well as growth hormone and nuclear receptor co-activators. Among others are ATM/RB-related pathways together with immune and inflammation signature including the antigen presentation pathway, OX40 signalling and cytotoxic T-lymphocyte-mediated apoptosis (Figure 6) (Curtis et al, 2012; Miller et al, 2011; Polyak, 2007; Shah et al, 2012). Moreover, genome-wide association studies highlighted that genetic modifications in three signalling pathways and one canonical signalling cascade, that are syndecan-1-mediated signalling,

hepatocyte growth factor receptor signalling, growth hormone signalling and the RAS/RAF/MAPK cascade, may contribute to breast cancer susceptibility (Menashe et al, 2010). Henceforth, the fact that mutated cancer genes are concentrated in only a limited number of pathways strongly signifies that targeting pathways rather than individual genes may be a more robust and effective therapeutic approach.

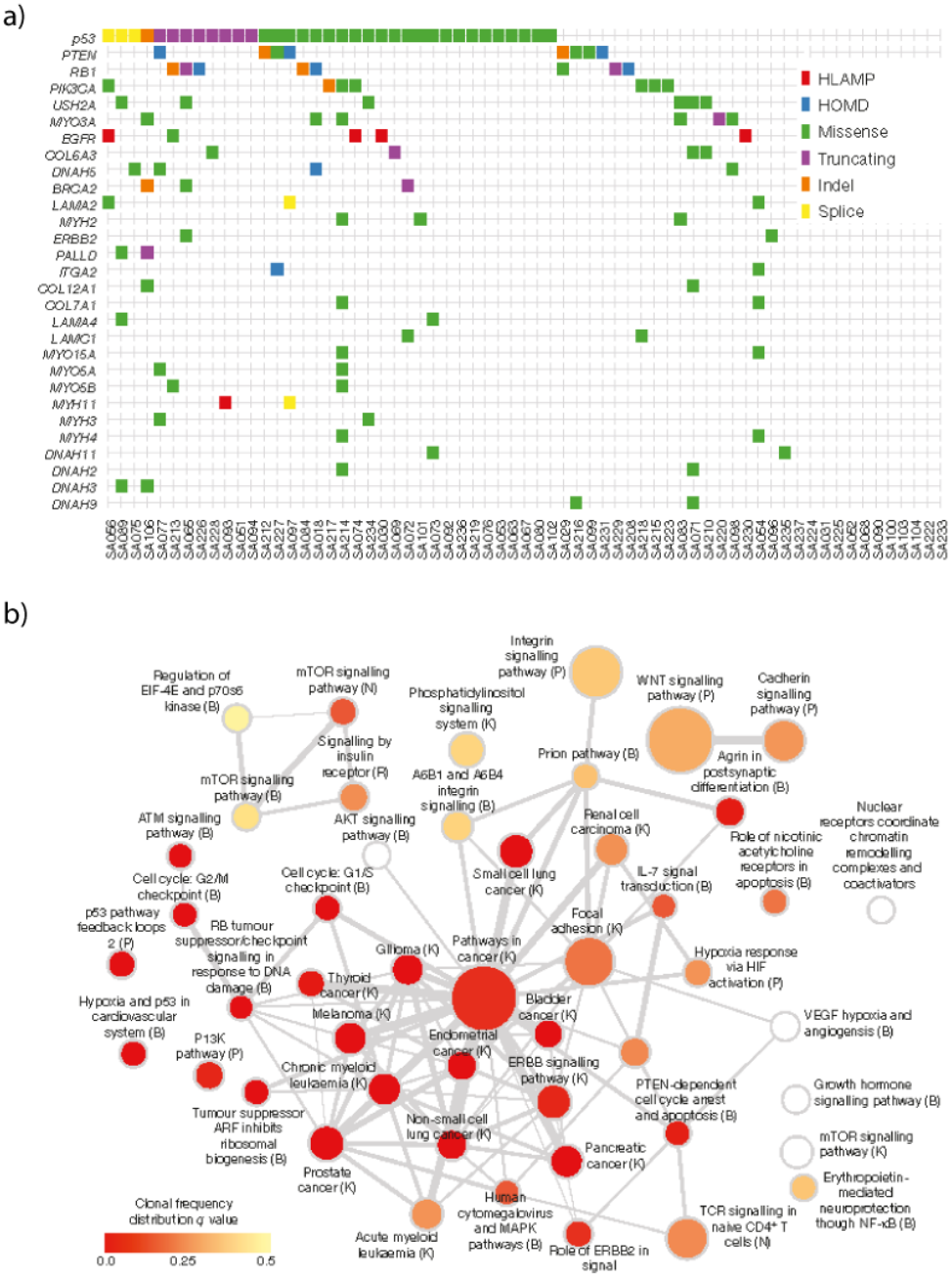

Figure 6. The clonal and mutational spectrum of primary triple-negative breast cancers

Adapted from (Shah et al, 2012). a) Mutually exclusive and co-occurring genomic abnormalities in triple-negative breast cancers (TNBC): Illustrated are case-specific mutations present in driver genes, in genes from integrin signalling and extracellular matrix (ECM)-related proteins (laminins, collagens, integrins, myosins and dynein) caused by all aberration types: high-level amplifications (HLAMP),

homozygous deletions (HOMD), missense, truncating, splice site and indel somatic mutations are represented in genes with at least two abnormalities in the population. b) Significantly overrepresented pathways (FDR<0.001) from 254 frequently mutated genes by somatic point mutations and indels are illustrated. Node shading encodes the adjusted *P* value (*q* value) of the comparison of the distribution of clonal frequencies of mutations in a certain pathway to the overall distribution of clonal frequencies. A broad range of clonal frequencies is evident (higher=red; lower=yellow). Database sources are indicated by letters in parentheses.

1.1.1.3 Classifications of breast tumour heterogeneity

The studies outlined above highlight the substantial genetic diversity underlying breast cancer. Indeed, breast cancer is not a monolithic entity but comprises heterogeneous tumours (Bertos & Park, 2011). Each breast cancer exhibits different clinical characteristics, disease course and response to specific treatments (Bertos & Park, 2011). Importantly, the number of somatic mutations as well as the combination of mutated genes varies markedly between individual breast tumours (Stephens et al, 2012). As a matter of fact, Stephens and colleagues noted that the maximum number of mutated cancer genes in an individual cancer was six, whereas 28 samples showed only one single driver (Stephens et al, 2012). Cells within a single tumour differ in size, morphology, antigen expression and membrane composition (Campbell and Polyak, 2007). They behave differently in terms of proliferation, cell-cell interaction, metastatic predisposition and treatment response (Campbell and Polyak, 2007). The genetic and epigenetic heterogeneity between tumours and within a single tumour, two concepts known as inter- and intra-tumour heterogeneity, complicates the research and treatment of breast cancer as characteristics of the most abundant cell type might not necessarily predict the properties of mixed populations (Campbell & Polyak, 2007; Dancey et al, 2012; Diaz-Cano, 2012; Marusyk et al, 2012; Schoenfeld et al, 2004). Therefore, it is desirable that a complete set of classifiers that can define an individual tumour comprising all the tumour-defining variables is established and applied to clinical trials of specific treatments. The development of such biological indicators will ultimately improve the stratification system in breast cancer patients and predict those patients that are most likely to respond to each potential intervention (Bernards, 2010; Dancey et al, 2012).

What are the breast tumour-defining variables? The list is rapidly expanding (Table 1) (Bertos & Park, 2011). Tumour-extrinsic features include microenvironment, whereas tumour-intrinsic features cover classical histological and immuno-pathological classifications along with molecular subtypes (Bertos & Park, 2011; Marusyk et al, 2012).

Classifier	**Classifications/variables**
Histological	IDC NOS, ILC, medullary, neuroendrocrine, tubular, apocrine, metaplastic, mucinous (A and B), inflammatory, comedo, adenocystic, micropapillary
Immunopathological	ER status, PR status, HER2 status
Transcriptional	Luminal A, luminal B, normal-like, basal/basal-like, HER2, claudin low, molecular apocrine
Genomic	17q12, basal complex, luminal simple, lumical complex, amplifier, mixed
Genomic heterogeneity	Monogenomic, polygenomic
miRNA-based	Multiple
Epigenetic	Multiple
Microenvironmental	Presence/activation status of local immune cells (T cells, B cells, dendritic cells, machrophages), fibroblast status, ECM composition, CAF status, angiogenesis, hypoxia
Macroenvironmental	Systemic hormone levels, BMI, overall immune status
Longitudinal	CTC features, metastatic features
Other	Intratumoural heterogeneity

Table 1. Selected breast tumour-defining variables contributing to diverse heterogeneity

Adapted from (Bertos & Park, 2011) **Confirmation Number: 11107126 Citation Information Order Detail ID:** 63848859 **The journal of clinical investigation by AMERICAN SOCIETY FOR CLINICAL INVESTIGATION Reproduced with permission of AMERICAN SOCIETY FOR CLINICAL INVESTIGATION in the format Republish in a thesis/dissertation via Copyright Clearance Center.**

The chief markers that are routinely used to assist therapy choice and serve as valuable predictors of therapy responsiveness in breast cancer are oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2; also known as ERBB2) (Baselga & Swain, 2009; Bast et al, 2001; Collins & Schnitt, 2005; Slamon et al, 1987). Combinations of these three biomarkers assign individual patients to specific clinical treatment groups with different overall prognosis: ER^+ ($ER^+/HER2^-$), $HER2^+$ ($ER^-/HER2^+$), triple-positive ($ER^+/PR^+/HER2^+$) and triple-negative ($ER^-/PR^-/HER2^-$).

Based on mRNA expression profiles, the ER^+ intrinsic group of tumours is mainly composed of two subtypes, namely luminal A and B, that constitute almost 80% of all breast cancers (Sorlie et al, 2001). Luminal subtypes exhibit the global expression pattern of breast luminal epithelial components. A major difference between the luminal groups is reflected in proliferation status that is low in luminal A and high in luminal B (Bertos & Park, 2011; Brenton et al, 2005; Perou et al, 2000). Consequently, luminal A tumours are associated with good prognosis, while luminal B patients exhibit poor prognosis (Table 2) (Andre et al, 2007). Fewer tumours carry mutations in *p53* and the majority is tumour grade I. The luminal A group is chemo-resistant and endocrine sensitive. In contrary, the luminal B type is endocrine less sensitive. In fact, it benefits more from a combination of chemotherapy and endocrine therapy.

ER^- cases contain predominantly three subtypes: a) normal-like subtype resembling normal epithelial tissue with large amount of non-tumour tissue, b) $HER2^+$ due to overexpression or excess gene copy number and c) basal or basal-like subtype corresponding to triple-negative breast cancer (TNBC) which counts for around 16% of all breast cancers (Blows et al, 2010; Perou et al, 2000). $HER2^+$ and basal-like cohorts display both a poor prognosis along with a higher risk for early relapse and show an overrepresentation of *TP53* mutations (Table 2) (Andre et al, 2007; Chae et al, 2009; Joshi et al, 2012). Basal-like breast cancers show expression patterns similar to normal breast myoepithelial cells as well as basal epithelial cells of other parts of the body. Importantly, they lack expression of HER2, ER and ER-related genes. In addition to tumoural X-inactivation, *BRCA1* mutations are associated with basal-like breast cancer risk and this BRCA-ness may have important therapeutic implications. From a prognostic point of view, $HER2^+$ patients benefit from molecularly targeted agents like anti-HER2 antibody trastuzumab. Nevertheless, given its triple-negative receptor status ($ER^-/PR^-/HER2^-$), basal-like breast cancer patients are considered to be not suitable for molecularly targeted therapies leaving genotoxic chemotherapy the so far only available treatment option (Dent et al., 2007).

	HER2$^+$/ER$^-$ tumour	Basal-like tumour	Luminal-like tumour A	Luminal-like tumour B
Poor prognosis	++	++	-	+
% of Tumour grade	8-80	80-91	15-20	50-55

Table 2. Clinical outcome of breast cancer molecular subclasses

Adapted from (Andre et al, 2007) **Confirmation Number: 11107130 Citation Information Order Detail ID: 63848872 Annals of oncology : official journal of the European Society for Medical Oncology by EUROPEAN SOCIETY FOR MEDICAL ONCOLOGY Reproduced with permission of OXFORD UNIVERSITY PRESS in the format reuse in a thesis/dissertation via Copyright Clearance Center.**

Intriguingly, recent integrated analyses of copy number and gene expression revolutionized our to some extent naive knowledge on the molecular stratification of the breast cancer population (Curtis et al, 2012). In fact, unsupervised analysis of paired DNA-RNA profiles revealed that breast cancer stems from 10 integrative clusters with distinct clinical outcomes and not only 5 as previously thought. Surely, this information forms the basis for next-generation treatments and help to better tailor therapeutic agents.

Interestingly, novel assessments of mutations, copy number and gene expression in triple-negative breast tumour samples unravelled that the term “triple-negative breast cancer” (TNBC), a tumour subtype first described by lack of oestrogen receptor (ER$^-$), progesterone receptor (PR$^-$) and ERBB2 gene amplification (HER2$^-$), is not a black-and-white model (Shah et al, 2012). Hence, these cases need further precise subclassifications. They are typically associated with expression of epidermal growth factor receptor (EGFR), high levels of proliferation markers such as Ki67, high cyclin E and low cyclin D1 levels as well as activated beta-catenin pathway (Arnedos et al, 2012). Moreover, although triple-negative breast cancer is used as a surrogate of basal-like breast cancer, there is some striking discordance caused by few HER2$^+$ and few ER$^+$ tumours within these basal-like cases (Hudis & Gianni, 2011). By next-generation sequencing mutational profiling analysis we now understand that triple-negative breast cancer samples are heterogeneous and at the time of diagnosis display a wide and continuous spectrum of genomic evolution between and within the tumours indicating that mutations can arise at multiple stages of tumour progression

(Figure 6) (Shah et al, 2012). Hence, it is of great importance that they should not be treated as a monolithic entity.

Together with the current clinical-pathological markers, the information provided by the newly identified heterogeneous entities further highlights the biological complexity of breast cancer (2012; Curtis et al, 2012; Prat & Perou, 2011; Shah et al, 2012). Overall, this combination provides a revolutionary comprehensive picture on molecular underpinnings of breast cancer and pinpoints avenues for treatment. Thus, this newly gained knowledge is likely to increase the efficacy of current and novel therapies and improve outcomes for breast cancer patients. In fact, it spotlights that at the time of primary diagnosis each patient and each tumour may display a very different stage of molecular progression and as a consequence will require different therapeutic implications (Shah et al, 2012). Indeed, the predictive value of a single biomarker could rely on the genetic background of the tumour and different breast cancer subtypes may exhibit different predictive markers of treatment responsiveness (Chae et al, 2009). Hence, determination of individual tumour clonal genotypes by studying hundreds to thousands of patients and their deregulated biology is necessary to achieve more durable patient responses.

1.2 Principle approaches to breast cancer treatment

To date, most advanced cancers remain incurable. If the removal of solid tumour masses such as by surgery is not a feasible option, the vast majority of the breast cancer treatment relies on a plethora of radiation, chemotherapy or hormonal or targeted therapy in an attempt to eliminate cancerous lesions (Marusyk et al, 2012).

Traditionally, standard chemotherapeutics have been identified mainly by empirical approaches, that is simply through trial and error (Chabner & Roberts, 2005). They reduce the cancer burden largely by killing rapidly dividing cells. Unfortunately, standard chemotherapy often causes collateral damage to healthy tissue leading to severe side effects that impair among others the circulatory system, immune system and digestive system. The main conceptual reason behind this is that the traditional drugs are not specific thus neither cancer cells nor the cancer causes are directly targeted. The traditional compounds usually affect processes in all rapidly dividing cells. Consequently, many normal healthy cells undergoing active growth and cell division can be also harmed. Furthermore, due to the severe side effects patients are

occasionally not able to take high enough doses thus cannot fight the cancer in the most effective way. Importantly, because of both intrinsic and acquired resistance only a minority of patients can usually benefit from such conventional therapies (Mullenders & Bernards, 2009). Furthermore, besides a high toxicity and a low quality of life, conventional therapeutics can give rise to therapy-induced secondary cancers (Voltz & Gronemeyer, 2008). Thus, standard chemotherapeutics display a relatively low therapeutic index that is a calculation of the therapeutic benefit relative to normal tissue toxicity for a given drug (Chan & Giaccia, 2008). From the clinical point of view, more potent, less toxic derivatives of standard chemotherapeutic agents exhibiting improved therapeutic indices and especially diverse chemotherapy schedules together with combinations have resulted in a better breast cancer control and therapy outcome (Ocana & Pandiella, 2008).

Besides non-selective chemotherapeutics, a treatment that targets directly the cause of cancer seems highly promising, as it should allow the specific elimination of the causal event. Surely, this requires cancer-causing molecular event to be identified and subsequently drug-targeted. Hence, such a so-called targeted therapy aims to directly inhibit the strength - referred to as Achilles' heel- of cancer cells by interfering with the activity of the deregulated oncogenic proteins that have become critical for the viability of tumour cells (Weinstein & Joe, 2006). The majority of targeted compounds so far inhibit mutationally activated kinases that the cell has become addicted to. Consequently, their sudden inhibition will result in cell death. This phenomenon is known as oncogene addiction (Luo et al, 2009b; McDermott et al, 2007; Weinstein & Joe, 2008). As they act on specific molecular targets, many targeted therapies result in no or just little collateral damage to healthy cells and thus cause fewer and less toxic side effects. Yet, the specificity of current targeted drugs shows one drawback. In fact, the inhibition of a single pathway in a tumour cell may be sufficient to slow it down yet often is not enough to kill the cancer. Therefore, many specific anti-cancer compounds are currently used in combination with standard chemotherapy (Table 3, Table 4) (NCCN; Ocana & Pandiella, 2008). The combined use of a highly specific cancer drug that is designed to only attack a tumour's Achilles' heel and traditional chemotherapy to deliver an effective assault on the tumour may prove to be a powerful means of fighting cancer.

CAF/FAC	Cyclophosphamide, doxorubicin, fluorouracil	Cytoxan, Adriamycin and 5-FU
FEC	Fluorouracil, epirubicin, cyclophosphamide	5-FU, Ellence and Cytoxan
AC	Doxorubicin, cyclophoshamide	Adriamycin and Cytoxan
EC	Epirubicin, docetaxel	Ellence and Cytoxan
AT	Doxorubicin, docetaxel	Adriamycin and Taxotere
CMF	Cyclophosphamide, methotrexate, fluorouricil	Cytoxan, Methotrexate and 5-FU
TC	Docetaxel, capecitabine	Taxotere and Xeloda
GT	Gemcitabine, taxol	Gemzar and Taxol

Table 3. Preferred chemotherapy combinations for breast cancer patients

Adapted from the NCCN Clinical Practice Guidelines in Oncology v.1.2012 (NCCN)

Subtype	**Treatment strategy**	**Molecular alterations**
Basal-like tumours	Chemotherapy + platinum compounds	DNA repair mechanism
	Anti-EGFR therapies (cetuximab)	EGFR expression
	c-Kit expression	Anti-Src therapies (dasatinib)
	Multiple small kinase inhibitors (sunitinib)	
Luminal tumours	Anti-oestrogen therapies	HR expression
	In combination with Herceptin in HER2-positive tumours	
HER2-positive tumours	Herceptin + chemotherapy	HER2 expression
	Herceptin + lapatinib	
	Herceptin + pertuzumab (after progression)	

Table 4. Promising future therapeutic strategies based on the patient genetic profile

Adapted from (Ocana & Pandiella, 2008). HR, hormonal receptor

Particularly, the examples for targeted treatment are ample in breast cancer. In several breast cancer forms aberrant hormonal and/or growth factor signalling play key roles in both tumour induction and resistance to treatment (Hanahan & Weinberg, 2000). The identification of molecular drivers in specific breast cancer

subtypes has led to the development of more efficacious forms of targeted therapy (Schechter et al, 1984; Slamon et al, 1987). Indeed, novel potential targets in breast cancer are being discovered based on cell biological and expression studies as is the case of receptors that mediate apoptosis, growth factors or their receptors (Ocana & Pandiella, 2008).

1.2.1 Chemotherapy responses in breast cancer

Even though intensive population screenings help us to obtain a detailed understanding of the molecular alterations, treatment response and cellular composition between and within tumours, breast cancer continues to steal annually 400.000 lives worldwide (Gonzalez-Angulo et al, 2007). A plausible explanation for this painful scenario is, in part, that we still lack an efficient enough translation of this information into therapies. Another major limiting factor for the effective cancer treatment is the molecular heterogeneity of this disease that is perfectly reflected in a diverse pattern of clinical responses (Park et al, 2010; Shipitsin et al, 2007). Furthermore, an additional major obstacle is represented by the unanticipated response to therapy as exemplified in breast cancer whereby only 1 in 30 post-menopausal women benefits from chemotherapy (2005; Mullenders & Bernards, 2009).

1.2.1.1 Chemotherapeutic sensitivity

Chemotherapy is a systemic treatment by disrupting DNA, interfering with protein production, preventing cell division, starving cells of nutrients or blocking hormone receptors of cancer cells. Current pre- and post-operative (neo-/ adjuvant) treatment strategies for breast cancer patients include several commonly used chemotherapy agents such as taxanes, anthracyclines, ixabepilone and platinum compounds (Hudis & Gianni, 2011). Combination regimens that include anthracyclines (epirubicin and doxorubicin) and alkylating agents (cyclophosphamide) or paclitaxel improve overall survival in patients with early breast cancer (2005; Loesch et al, 2010; Mauri et al, 2005; Rivera & Gomez, 2010). Many hormone receptor positive (HR^+) patients initially respond to hormone ablation therapy such as aromatase inhibitors that stop oestrogen production in post-menopausal women or receptor antagonists like tamoxifen (Chia et al, 2010; Ellis et al, 2012). Despite the survival benefit due to chemotherapeutics, metastatic breast cancer remains incurable, patients displaying a

median survival between one and two years (Gonzalez-Angulo et al, 2007; Jones, 2008; Whitehurst et al, 2007).

Chemotherapeutics effectively applied in breast cancer patients over significant periods of time and their efficacy in different breast cancer molecular subtypes are summarized in Table 5 and Table 6. Strikingly, the molecular underpinnings of clinically relevant DNA-alkylating or -intercalating tool compounds, topoisomerase inhibitors along with the selectivity profiles of anti-metabolites for transformed cells are not comprehensively explored yet (Danial & Korsmeyer, 2004; Hartwell et al, 1997; Schmitt & Lowe, 1999). Even though some drug mechanisms are elucidated in detail, we do not exactly understand how chemotherapeutics induce tumour regression (Hartwell et al, 1997). What we have learnt to appreciate is, however, that our simplistic conviction that tumour cells are sensitive to chemotherapy just because they rapidly divide, do not reflect the entire story (Smida & Nijman, 2012). For instance, it is thought that DNA damaging drugs attack cancer cells due to already present defects in DNA repair and checkpoint which in turn ultimately stimulate pro-apoptotic signals or lead to mitotic catastrophe (Kaelin, 2009). Moreover, detoxifying enzymes, drug efflux pumps, aneuploidy along with metabolic states belong all to potential contributors of the chemotherapy response (Bosch, 2008; Chang, 2011; Sauna et al, 2007; Smida & Nijman, 2012).

Drug	Mode of action	Effect
Paclitaxel **Docetaxel** **Vinorelbine**	bind to β-tubulin inhibit spindle-microtubule dynamics impair mitotic spindle assembly	Cytotoxic
Doxorubicin **Epirubicin**	inhibit DNA topoisomerase II intercalate in DNA alkylate and cross-link DNA induce free radical formation	Genotoxic
Capecitabine	affects pyrimidine synthesis inhibits thymidylate synthase	Genotoxic
Cyclophosphamide	alkylates DNA cross-links DNA causes strand breaks induces mutations	Genotoxic
Tamoxifen **4-Hydroxy-tamoxifen** **Fulvestrant**	selective oestrogen receptor modulator (SERM) inhibit Protein Kinase C (PKC) impair VEGF production inhibit lipid peroxidation	

Table 5. Common chemotherapeutics in breast cancer treatment

Docetaxel is a semi-synthetic analogue of paclitaxel (Jordan & Wilson, 2004), whereas epirubicin is a semi-synthetic derivative of doxorubicin (Bonadonna et al, 1993). In the liver, capecitabine is converted to fluorouracil (Rivera & Gomez, 2010). Fulvestrant structurally differs from tamoxifen and 4-hydroxytamoxifen (Osborne et al, 2004). The evidence for genotoxic mechanisms of tamoxifen carcinogenicity is inconsistent (Phillips, 2001).

Response to therapy	$HER2^+/ER^-$ tumours	Basal-like tumour	Luminal-like tumour
Tamoxifen	-	-	++
Anthracycline-based chemotherapy	+++	++	+/-
Paclitaxel	+++	++	+/-
Trastuzumab	+++	-	+ (subset of patients with $HER2^+/ER^+$ tumours)

Table 6. Efficacy of therapies depending on breast cancer genetic profile subclasses

Adapted from (Andre et al, 2007) **Confirmation Number: 11107130 Citation Information Order Detail ID:** 63848872 **Annals of oncology : official journal of the European Society for Medical Oncology**

1.2.1.2 Chemotherapeutic resistance

Despite significant progress in the application of cancer chemotherapy over the last 50 years, most chemotherapeutic treatments ultimately fail to cure patients (Holen & Saltz, 2001). Even tumours that respond to therapy in the early stages frequently relapse as chemo-resistant malignancies. This is thought to be, in part, due to passengers in minor subpopulations wherein the admission of a respective therapy changes the tumour environment. As a consequence, the passenger is converted to a driver, manifesting as the recurrence (Mullighan et al, 2008; Roche-Lestienne et al, 2002; Stratton et al, 2009). Overall, the efficacy of cancer therapy is invariably limited by innate or acquired drug resistance, that is, either prior to drug treatment or over time in response to persistent drug exposure, respectively. Strikingly, some patients may also acquire multidrug resistance to structurally unrelated agents even though they were exposed to a single chemotherapeutic compound only (Abolhoda et al, 1999; Di Nicolantonio et al, 2005; Giaccone & Pinedo, 1996; Rivera & Gomez, 2010).

Here, an interesting question is: When does chemo-resistance occur? Actually, it may arise rapidly in response to drug application (Di Nicolantonio et al, 2005; Giaccone & Pinedo, 1996; Rivera & Gomez, 2010) and as a consequence to various defects in transformed cells (O'Connor et al, 2007). Therapeutic resistance to Herceptin or lapatinib, regardless of admission as either single agents or in combination with chemotherapy in a metastatic setting, for instance, typically occurs within months after starting therapy (Garrett & Arteaga, 2011). Remarkably, studies on cancer biopsies have indicated the occurrence of resistance being within hours or days upon drug exposure *in vitro* and *in vivo* (Abolhoda et al, 1999; Di Nicolantonio et al, 2005).

Another important notion we need to understand is: How does drug resistance arise? Blessedly, mechanisms of acquired drug resistance are beginning to be elucidated through the molecular analysis of resistant specimens (Table 7) (Engelman & Janne, 2008; Engelman & Settleman, 2008; Garraway & Janne, 2012; Guix et al, 2008). A likely mechanism of acquired resistance is thought to be the decreased drug bioavailability due to increased drug efflux or plasma sequestration (Apperley, 2007;

Gambacorti-Passerini et al, 2000; Melo & Chuah, 2007). Indeed, among the best-characterized resistance mechanisms are drug efflux pumps such as components of ATP-dependent membrane transporters and ABC drug transporters (Di Nicolantonio et al, 2005; Giaccone & Pinedo, 1996; Rivera & Gomez, 2010).

Secondary genetic alteration in drug target
Mutation in drug contact residue
Mutation in non-contact residue leading to altered conformation
Mutation leading to increased ATP affinity
Amplification
Alternative spliced form
Bypass mechanism
Activation of parallel signalling pathway
Alterations in upstream or downstream effectors
Upstream effector
Downstream effector
Pathway independent
Epithelial-mesenchymal transition
Changes in tumour microenvironment
Altered angiogenesis

Table 7. Acquired drug resistance mechanisms

Adapted from (Garraway & Janne, 2012)

We now comprehend that the increasingly appreciated heterogeneity of tumours and their high mutation rates contribute and facilitate the rapid selection for drug-resistant cells and thus further complicates the elucidation of drug resistance mechanisms (Calbo et al, 2011; Quintana et al, 2010; Sharma et al, 2010). In the case of kinase inhibitors, for instance, the frequent acquisition of a secondary mutation at the drug-binding site interferes with the inhibition of kinase activity upon drug exposure (Kobayashi et al, 2005; Pao et al, 2005). Moreover, cell intrinsic genetic alterations such as activation of detoxifying enzymes or apoptotic defects as well as extrinsic factors including cytokines and growth factors have been suggested to contribute to chemo-resistance (Eckstein et al, 2009; Williams et al, 2007; Wilson et al, 2012). It is also important to mention that tumour microenvironment with its associated stromal, immune and endothelial cells adds up to the complexity of chemotherapeutic outcome (Hanahan & Weinberg, 2000). Recent studies demonstrated that DNA

damage-inducing genotoxic drugs can create a chemo-resistant niche in favoured anatomical sites that initiates the survival of a minimal residual tumour burden and serves as a supply for potential tumour relapse (Gilbert & Hemann, 2010; Liang et al, 2010). In addition, another factor has been recently implicated as the source of eventual tumour relapse: the presence of rare cancer stem cells with the capability of self-renewal and differentiation. This cell subpopulation is thought to be resistant to treatment due to increased genomic stability, decreased oxidative stress or the presence of multiple drug resistance transporters (Gilbert & Hemann, 2010; Visvader & Lindeman, 2008). Importantly, clinical analyses have discovered a correlation between the proportion of breast cancer stem cells and poor prognosis (Velasco-Velazquez et al, 2012). Indeed, in this context, a significant effort has been made to uncover the oncogenic alterations leading to the initiation and/or maintenance of the cancer stem cell subpopulation (Al-Hajj et al, 2003; Bjerkvig et al, 2005; Weissman, 2005).

Remarkably, despite many studies on commonly deregulated prominent cancer genes and their association with chemotherapy response, results have been highly controversial. In fact, amplification of c-MYC, for instance, has been shown to render both drug sensitivity and resistance under respective conditions (Bidwelliii & Raucher, 2006; Bottone et al, 2003; Brennan et al, 1991; Deming et al, 2000; Ernberg et al, 2009; Grassilli, 2004; Johnson et al, 1996; Nakagawara et al, 1990; Park et al, 2002). Although p53 is considered as an established specific prognostic factor in breast cancer patients, some reports have associated p53 overexpression with both sensitivity and resistance to neoadjuvant anthracycline-based chemotherapy, whereas others observed no association (Berns et al, 2000; Bottini et al, 2000; Chae et al, 2009; Geisler et al, 2001; Kandioler-Eckersberger et al, 2000; MacGrogan et al, 1996; Makris et al, 1997; Mathieu et al, 1995; Niskanen et al, 1997; Rozan et al, 1998). However, there is no unique explanation to account for these inconsistencies. Yet, some could be surely justified by methodical differences in how p53 mutation status is determined, by variations in tumour samples, patient treatment, cohort size, distinct cellular contexts and genetic make-up of individual breast tumours as well as great heterogeneity among patients (Alsner et al, 2000; Berns et al, 2000; Colleoni et al, 1999; Di Nicolantonio et al, 2005; Rivera & Gomez, 2010).

Altogether, all these reports spotlight and remind us the urgent need for predictive biomarkers since conventional biomarkers such as particular histo-pathologic

characteristics are not accurate enough to forecast chemotherapy responsiveness (Deming et al, 2000).

1.2.2 Targeted therapy and smart drugs: too few success stories

One major focus of current breast cancer research is the identification of biomarkers to anticipate the drug responsiveness and the design and development of specific anti-cancer drugs to treat individual patients wherein the treatment selection is driven by a detailed knowledge of the genetics and biology of the patient and their disease (de Bono & Ashworth, 2010). Indeed, the emphasis in drug development is no longer on non-specific, cytotoxic chemotherapies but on molecularly targeted and rationally designed compounds ultimately promising greater efficacy and fewer side effects. Ideally, targeted agents display high therapeutic indices to selectively kill tumour cells without harming other cells of the body.

Generally, one distinguishes between two classes of targeted agents: monoclonal antibodies and small molecules including peptides, nucleic acids, lipids, carbohydrates and other bioactive drugs (Chan & Giaccia, 2008).

Herceptin (trastuzumab) for the treatment of metastatic breast cancer represents the first example of genomic research-based targeted anti-kinase therapeutic approved in the late 1990s. This monoclonal antibody was designed to target HER2 receptor tyrosine kinase in breast cancer cells (Chan & Giaccia, 2008; Goldenberg, 1999; Pegram et al, 1999). Here, it is important to remember that approximately one quarter of breast cancer patients display increased levels of HER2 and are thus associated with higher malignancy and poor prognosis. Upon binding of the humanized monoclonal antibody trastuzumab to the extracellular domain of the HER2 receptor, downstream signalling abolishes, consequently cells undergo cell cycle arrest (Chan & Giaccia, 2008; Mukai, 2010). Henceforth, Herceptin represents a prototype for the development of a targeted therapy. Despite the initial success cases for the treatment of $HER2^{+}$ breast cancer patients, however, the majority of metastatic breast cancers develop acquired tolerance and a large cohort does not respond at all (Mukai, 2010; Ocana & Pandiella, 2008; Shawver et al, 2002).

Other HER1 and/or HER2 targeting humanized monoclonal antibodies as well as those designed against more general features of cancers are either in clinical application or under development for breast cancer treatment (Ferrara, 2002; Jain, 2002; Mukai, 2010). Pertuzumab, for instance, has been raised as an anti-HER2 antibody based on the subdomain II of HER2. In contrast to trastuzumab, it blocks the activation of HER receptors upon ligand stimulation. Numerous clinical trials are investigating its activity in $HER2^{+}$ cancer patients either a monotherapy or in combination with other targeted agents (Ocana & Pandiella, 2008).

Strikingly, neither Herceptin nor any other specific antibodies proven to be effective in leukemia or lung cancer have cured individuals with advanced disease. Yet, they can substantially prolong life and improve its quality and thus definitely represent a promising tool to specifically attack cancer cells with transformed traits (Vogelstein & Kinzler, 2004). Indeed, demonstration of the anti-tumour activity of Herceptin in breast cancer patients served as the proof of principle for targeted therapy directed against receptor tyrosine kinases, a class of cell surface receptors (i.e HER2, EGFR, IGF-1R, PDGFR, CSF-1R, c-kit, Flt 4, FGFR, Met and Tie-2), each one being a potential drug target (Table 8) (Ocana & Pandiella, 2008; Shawver et al, 2002).

Target	Drug
ErbB receptors	Antibodies
	Pertuzumab (Omnitarg; Genentech)
	Small tyrosine kinase inhibitors
	Dual ErbB1-HER2
	Lapatinib (GlaxoSmithKline)
	EKB-569 (Wyeth)
	HER2 CP-724714 (Pfizer)
IGF-IRs	Small tyrosine kinase inhibitors
	NVP-AEW541 (Novartis)
	BMS-536924 (Bristol)
	Antibodies
	IMC-A12 (Imclone)
	AMG 479 (Amgen)
	CP 751,871 (Pfizer)
PI3K/AKT pathway	PI3K inhibitors
	SF1126 (Semafore)
	mTOR inhibitors
	Everolimus (RAD001; Novartis)
	Temsirolimus (CCI-779; Wyeth)
	AP23573 (Ariad)
	AP23841 (Ariad)
Src inhibitors	Dasatinib (Bristol-Myers Squibb)
	AZD0530 (AstraZeneca)
	Bosutinib (SKI-606)
HDAC inhibitors	SAHA (Merck)
	CI-994
	LBH-589 (Novartis)
Proteasome pathway	Proteasome inhibitors
	Velcade (Millenium)
Chaperone inhibitors	17AAG (KOS-953)
Angiogenesis	Anti-VEGF bevacizumab (Avastin; Roche)
	VEGF-Trap
	Multityrosine kinase inhibitors
	VEGFR2 (sunitinib, Sutent; Pfizer)
	Sorafenib (Bayer)
	VEGFR2-MET (XL880; Exelixis)
	VEGFRs (vatalanib; Novartis)
	Antibodies anti-VEGFRs

Table 8. Selected druggable oncogenic pathways and treatment options in breast cancer

Adapted from (Ocana & Pandiella, 2008). mTOR, mammalian target of rapamycin; 17AAG, 17-allylamino-17-demethoxygeldanamycin; HDAC, histone deacetylase; SAHA, suberoylanilide hydroxamic acid

On the other hand, the most prominent small molecule inhibitor applied to fight numerous cancers discovered by gene-based *in vitro* screenings is Gleevec (imatinib mesylate). This competitive tyrosine kinase inhibitor is originally identified to treat chronic myeloid leukemia characterized by the oncogenic BCR-ABL chromosomal translocation (Bryant et al, 2005; Druker et al, 1996; O'Hare et al, 2006). Importantly, in the last years Gleevec has been effective in gastrointestinal stromal tumours and some other cancers including metastatic breast cancer that rely on related receptor tyrosine kinases such as c-kit (CD117) and PDGFR-α (Chan & Giaccia, 2008; Cristofanilli et al, 2008; Druker et al, 1996).

In addition, even though it remains to be FDA-approved, clinical phase II studies for the EGFR tyrosine kinase inhibitior Iressa (gefitinib) that has mainly been used in non-small cell lung cancer treatment are currently underway proving its power to treat metastatic breast cancer patients in combination with traditional chemotherapeutics (Bernsdorf et al, 2011). It is also encouraging that other preclinical data report that when combined with platinum compounds, anti-EGFR therapies are proven to be extremely active (Ocana & Pandiella, 2008). This is why current clinical trials are focusing on the effect of DNA damaging agents combined with anti-EGFR therapies. Previously, a neoadjuvant cisplatin clinical trial in phase II has reported a good clinical activity as a monotherapy in a triple-negative cohort of patients (Silver et al, 2010). Moreover, a novel class of tyrosine kinase inhibitors with a dual EGFR/HER2 activity, called lapatinib, has exhibited promising outcomes (Burris et al, 2005).

Besides membrane receptors, cytosolic events also represent attractive targets for drug development in breast cancer research. Among such pathways are RAS and PI3K signalling cascades. Even though in contrast to other tumour types oncogenic RAS mutations are rare in breast cancer (Barbacid, 1987), different drugs have been developed to target this particular signalling pathway such as farnesyltransferase inhibitor, RAF inhibitors and MAPK/extracellular signal-regulated kinase inhibitors (Figure 7) (Ocana & Pandiella, 2008). In addition, a wide spectrum of PI3K pathway inhibitors is in clinical development or entering into phase III trials (Figure 7, Table 8) (Ocana & Pandiella, 2008; Rodon et al, 2013).

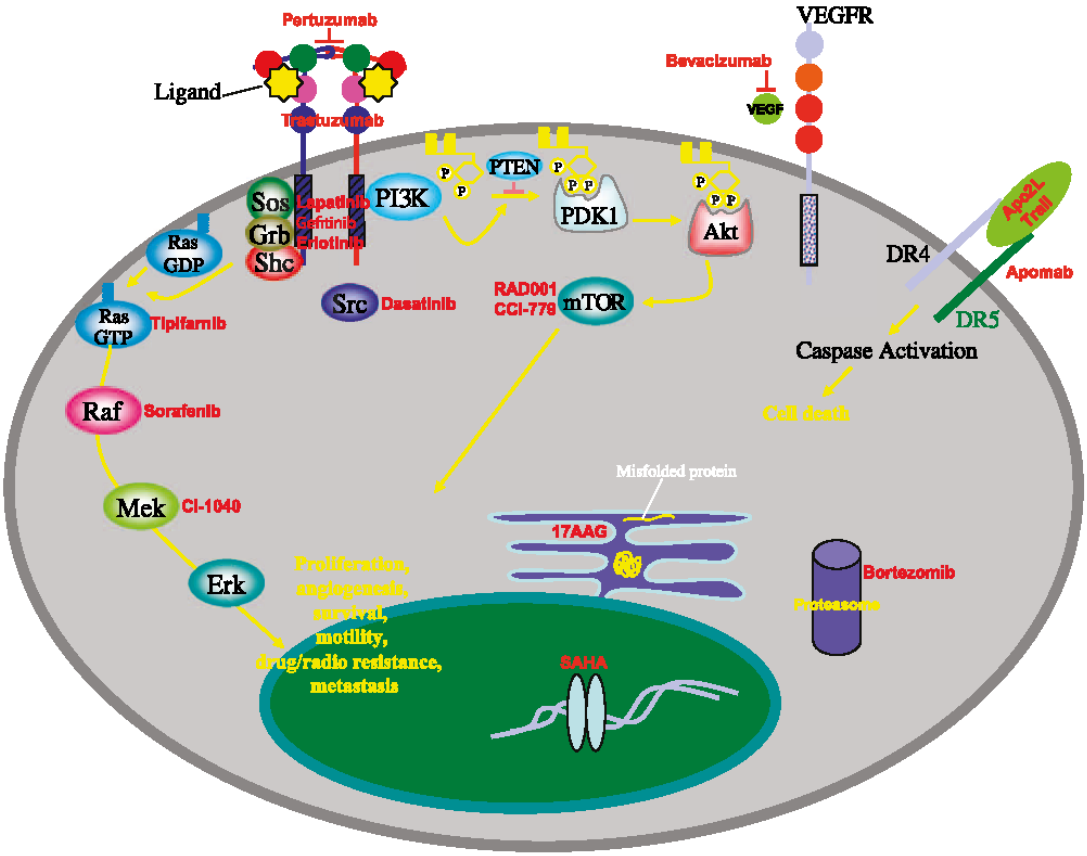

Figure 7. Targeted druggability of the intracellular signalling pathways

Adapted from (Ocana & Pandiella, 2008). mTOR, mammalian target of rapamycin; Mek, MAPK/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; 17AAG, 17-allylamino-17-demethoxygeldanamycin

Moreover, particular attention in breast cancer research is currently paid to a number of inhibitors attacking the chaperone machinery, the ubiquitin-proteasome pathway and epigenetics along with inhibitors for vascular endothelial growth factor receptor, Src kinase and checkpoint kinase 1 being under development (Table 4, Table 8) (Hudis & Gianni, 2011; Ocana & Pandiella, 2008; Shawver et al, 2002). In fact, the combination of an anti-angiogenic monoclonal antibody targeting vascular endothelial growth factor (VEGF) A (Bevacuzimab/Avastin) together with conventional chemotherapy has recently demonstrated to improve the progression-free survival of HR^+ and $HER2^-$ patients (Bear et al, 2012; Miller et al, 2005).

1.3 General concept and utility of synthetic lethality to define novel breast cancer agents

Even though exploiting loss-of-function mutations or gene deletions has so far not been such a great success, the studies introduced above highlight the encouraging clinical progress that has been achieved by targeted drugs that are directed against amplified or mutationally activated key driver oncoproteins that are often kinases. Nevertheless, the overall number of success stories in the development of targeted therapies is too few mainly because a) suitable targets for cancer therapy are difficult to find and b) transforming our knowledge on specific molecular alterations of cancer cells into targeted therapies is laborious (Lord and Ashworth, 2010; Pegram et al., 2004; Yang et al., 2011; Vogel et al., 2002).

What does hamper the development of highly selective targeted drugs (Kaelin, 2005; Lord & Ashworth, 2010; Mullenders & Bernards, 2009; Sellers, 2011)? First of all, what we have to keep in mind is that most small molecule compounds inactivate proteins by binding to the catalytic site of an enzyme. As a consequence, the drug development is restricted to the products of oncogenes that are hyperactive in cancer cells due to activating mutations or overexpression but not to tumour suppressor genes. In fact, mutations inactivating tumour suppressor genes and thereby yielding complete loss of their protein products present a major therapeutic challenge since most drugs also inactivate but not reactivate their targets. In addition, many prominent driver oncogenes such as c-MYC and ETS are not considered to be „druggable", that is when a low-molecular-weight compound readily inhibits the protein activity, or they are found only in a small fraction of tumours limiting the clinical utility. Moreover, many of the hyperactive breast cancer genes play key roles in healthy cells thus offering only a limited therapeutic window.

An elegant solution to overcome all these hurdles has been provided by a concept from classical genetics termed as synthetic lethality. Translated from the ancient Greek, synthetic means here the combination of two entities to form something new (Nijman, 2011). This phenomenon is also known as non-oncogene addiction or induced essentiality (Luo et al, 2009b; Tischler et al, 2008). The history of synthetic lethality is over 70 years old and was introduced by Calvin Bridges and Theodore Dobzhansky who observed by crossing fruit flies -*Drosophila melanogaster* or *pseudoobscura*, respectively- that certain non-allelic genes were lethal only in

combination, although the homozygous parents were viable (Bridges, 1922; Dobzhansky, 1946; Nijman, 2011).

This concept thus describes the relationship between two genes and takes advantage of the notion that some genes are only lethal to cells if a second non-lethal mutation is also present (Kaelin, 2005; Kaelin, 2009; Luo et al, 2009b; Nijman, 2011; Tong et al, 2001). Thus, if gene *A* and gene *B* have a synthetic lethal interaction, inactivation of either gene *A* or gene *B* will not impair viability. Only inactivation of both genes will result in a lethal phenotype. In addition, a non-lethal growth impairment can also arise upon any combination of perturbations which is referred to as synthetic sickness that is usually categorized under synthetic lethal interactions (Nijman, 2011). Though best studied within loss-of-function mutations, synthetic lethality can also be observed upon any combination of cellular perturbation such as overexpression of an oncogene, action of a chemical compound or environmental change, the underlying principle being that two perturbations might change the requirement of a third one (Figure 8) (Hillenmeyer et al, 2008; Kaelin, 2009; Lehar et al, 2008; Luo et al, 2009b; Nijman, 2011).

Gene *A*	Gene *B*	
A	*B*	Viable
a	*B*	Viable
A	*b*	Viable
a	*b*	Lethal

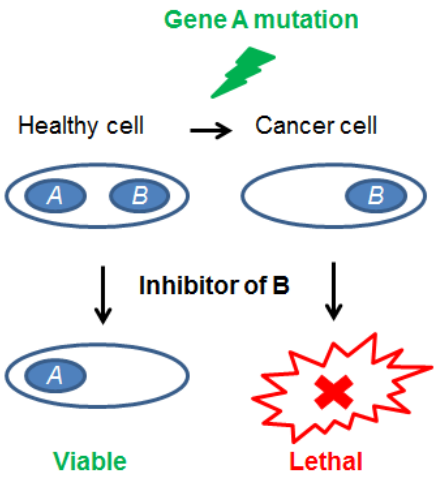

Figure 8. The concept of synthetic lethality upon genetic and chemical perturbations

Lower case, mutant; upper case, wild type

The notion that synthetic lethal interactions are common in biology has been successfully demonstrated in large-scale genetic screens in model organisms such as the yeast *S. cerevisiae* and the worm *C. elegans* where up to 1% of all randomly picked gene pairs displays a synthetic lethal phenotype (Lehner et al, 2006; Nijman, 2011; Schuldiner et al, 2005). Each yeast gene in fact has on average 30 synthetic sick or lethal interactions with other genes. Furthermore, it has been documented that only 20% of the genes are individually essential, whereas synthetic lethal interactions are found to be common among the remaining 80% (Kaelin, 2009; Nijman, 2011; Sharom, 2004; Tong, 2001; Tong, 2004). In addition, synthetic lethal analysis of each single yeast gene has successfully guided the decipherment of the function of individual proteins, while a global analysis holds promise for a comprehensive view of the pathways that can buffer each other biologically (Nijman, 2011; Ooi et al, 2006).

Importantly, in the last years synthetic sickness/lethality has become more and more of a concept that can be applied to exploit cellular weak spots caused by genetic or epigenetic aberrations in cancer cells. As such interactions in tumour cells hold great promise for the development of more rational, effective and specific therapies, it is important to emphasize that the therapeutic exploitation of synthetic lethal interactions in cancer cells is fundamentally different than therapies targeted against a tumour's strength. We also expect that exploiting a tumour's weak spot by targeting the synthetic lethal partner may act complementary and even synergistically to currently existing therapeutic interventions (Nijman, 2011). In fact, compounds that disrupt the function of pathways parallel to the loss of a tumour suppressor gene may result in cell death specifically in transformed cells (Bommi-Reddy et al, 2008; Chan & Giaccia, 2008; Hartwell et al, 1997; Kaelin, 2005). In a similar way, hyperactive oncogenes could sensitize cells to certain compounds due to newly arisen dependencies on particular pathways (Ji et al, 2009; Yang & Stockwell, 2008). Indeed, we understand that all cancerous aberrations, being either drivers responsible for the transformed phenotype or passengers, hold the potential to shift the cellular requirement for a certain target and hence contribute to selectivity (Bommi-Reddy et al, 2008; Chan & Giaccia, 2008; Hartwell et al, 1997; Kaelin, 1999; Kaelin, 2005; Reddy & Kaelin, 2002). Recent intriguing work in the area of gene deletions proposed a potential strategy that exploits passenger mutations (Lehner & Park, 2012; Muller et al, 2012). In this conceptual framework, such mutations can remove or inactivate genetic redundancies in cancer cells, generating cellular weak spots and so facilitating their exquisitely selective destruction.

1.3.1 Examples of how to directly drug the undruggable in breast cancer

Synthetic lethal approaches therefore provide a framework for pharmacologically challenging targets that are not classically druggable such as proteins that cannot be targeted with enzymatic, active-site inhibitors as well as the complete loss of tumour suppressor (Bommi-Reddy et al., 2008; Dolma et al., 2003; Hartwell et al., 1997; Ji et al., 2009; Kaelin, 2005; Yang and Stockwell, 2008). In fact, synthetic lethal interactions in cancer cells are starting to emerge (Schlabach et al, 2008; Silva et al, 2008; Whitehurst et al, 2007).

The most promising synthetic lethal interaction that has made its way to the clinic for breast cancer patients was observed between mutations in *BRCA1* or *BRCA2* and pharmacological inhibition of poly-adenosine diphosphate (ADP)-ribose polymerases (PARP) (Bryant et al, 2005; Farmer et al, 2005).

Applying the synthetic lethality concept, how was the driving hypothesis built? We know that the presence of *BRCA* mutation and subsequent non-functional homologous recombination alone are not sufficient to result in tumour cell death. Keeping the idea of synthetic lethality in mind, however, the inhibition of an additional DNA repair pathway with a PARP inhibitor could lead to the death of *BRCA*-deficient tumours. Explicitly, it is believed that PARP1 inhibition causes persistence of single-strand DNA breaks, resulting in replication fork collapse and accumulation of potentially lethal double-strand breaks. Indeed, this hypothesis was proven whereby in *BRCA*-deficient cells PARP inhibitors were up to 1000-fold more effective compared to isogenically matched *BRCA*-proficient cells, providing a remarkable selectivity window (Farmer et al, 2005; Ratnam & Low, 2007).

Moreover, enhanced sensitivity to PARP inhibition has also been seen in cells harbouring defects in homologous recombination other than *BRCA* deficiency (McCabe et al, 2006; Mendes-Pereira et al, 2009; Sourisseau et al, 2010; Weston et al, 2010; Williamson et al, 2010). This suggests that targeting compensatory DNA repair pathways could permit tailoring of chemo- or radiotherapy to the specific genetic background of the individual tumours (Aggarwal & Brosh, 2009). Thereby, therapeutic indices of classical anti-breast cancer treatments could be expanded and side effects reduced (Ratnam & Low, 2007).

It is worth noting that besides as a monotherapy in patients with *BRCA1/2* mutations, PARP inhibitors are being investigated as a strategy to potentiate the DNA damaging effects of conventional chemo- and radiotherapy as well as in the treatment of triple-negative breast cancer due to its molecular similarities to *BRCA1*-mutated malignancies (Beneke et al, 2004; Calabrese et al, 2004; Graziani & Szabo, 2005; Tentori et al, 2002). In fact, women with triple-negative tumours and concurrent *BRCA* mutation show a better prognosis and thus may respond to PARP inhibitors (Fong et al, 2009; Fong et al, 2010).

Of particular interest, clinical phase I trial data of *BRCA1*- or *BRCA2*-deficient breast cancer patients administered the oral PARP1 inhibitor olaparib were initially very encouraging (Fong et al, 2009). Strikingly, however, one pilot trial with olaparib did not yield tumour responses in breast cancers regardless of *BRCA* status thus this drug is not currently developed for breast cancer treatment (Tripathy, 2011). Furthermore, trials with two other PARP inhibitors, AGO14699 and MK4827, as single agents also exhibited activity in *BRCA1*- or *BRCA2*-related tumours but additionally, activity was also observed with MK4827 in sporadic cancers (Tripathy, 2011). On the other hand, iniparib, which is now known to be not a strong PARP1 inhibitor, created significant enthusiasm when a randomized phase II trial demonstrated a dramatic survival advantage (O'Shaughnessy et al, 2011). The follow-up phase III trial, however, did not meet its objectives (Tripathy, 2011). This points out that early trials may not be large enough to drive definite conclusions yet in addition, several other factors might also account for the observed discrepancies (Rios & Puhalla, 2011). Still, despite the challenges in these early stages and pressing questions waiting to be answered, this class of agents has illustrated the bench-to-bedside potential of individualized therapies and emphasized that it is critical to design accurate correlative studies to define the optimal regimens and candidate populations (Rios & Puhalla, 2011).

Altogether, the synthetic lethality between BRCA and PARP qualifies as a prime example for a tumour's vulnerability due to loss of a tumour suppressor gene. In addition, among the specific oncogenic lesions in mammalian cells that have been surveyed, a particular attention was paid on targeting the loss of the *VHL* tumour suppressor (Bommi-Reddy et al, 2008) and the activated *KRAS* oncogene (Luo et al, 2009a). Interestingly, studies reported promising targets including the mitosis checkpoint protein Polo-like kinase 1 (PLK1) and survivin. In fact, clinical trials using

small molecule inhibitors against PLK1 (BI02536) and survivin (YM155) are ongoing (Mross et al, 2008; Satoh et al, 2009).

Nevertheless, synthetic lethal interactions were also observed between oncogenes and small compounds but these remain to be validated in a clinical setting. Cells with an amplified MYC, for instance, displayed hypersensitivity to apoptosis induced by death receptor ligands (Rottmann et al, 2005; Wang et al, 2004). Moreover, cancer cells with activated RAS^{V12} conferred enhanced sensitivity to erastin, a compound causing oxidative, non-apoptotic cell death in a RAS-RAF-MEK-dependent manner (Yang & Stockwell, 2008).

Synthetic lethal screening efforts ultimately aiming to define mutations that render cells more sensitive to chemotherapeutics have thus far resulted in few yet potentially clinical exploitable interactions (Mizuarai et al, 2008). The knock-down of a collection of genes including CT-antigen family members and proteasome subunits, for instance, increased responsiveness to paclitaxel in a human non-small cell lung cancer cell line (Whitehurst et al, 2007). Another screen for knock-downs causing hypersensitivity to treatment with paclitaxel, cisplatin and gemcitabine uncovered that disruptions in BRCA1/2 pathway components and *TP53* together enhanced cisplatin cytotoxicity in various cell lines than either perturbation alone, whereas other proteins such as ribonucleotide reductase subunit M1 exhibited synthetic lethality in *p53*-deficient cells upon exposure to gemcitabine (Bartz et al, 2006). However, to entirely explore the number of synthetic lethalities with chemotherapeutics in tumour cells, advanced unbiased and systematic screening approaches will be required as such synthetic lethal interactions are actually rare and certainly not easy to forecast (Kaelin, 2009; Nijman, 2011).

1.3.2 Genetic screening tools

To date, an array of advanced technologies including DNA sequencing, comparative genomic hybridization, single-nucleotide polymorphism genotyping, proteomics and gene expression microarrays have all been implemented in drug development efforts. The main drawback, however, is that the data obtained are mostly correlative and as a consequence may not directly reflect the driver event among the many genetic alterations present in each cancer (Mullenders & Bernards, 2009). Of interest, loss-of-function screens hold great promise for the direct identification of

causal factors only. Indeed, such screening efforts are more suitable for discovery of drug targets as the genetic event mimics the intended effect of the cancer drug: reduced gene product activity.

In the last years, unbiased screening approaches in sophisticated high-throughput formats with flexible assay design and fairly low-costs have become available (Nijman, 2011; Schlabach et al, 2008; Silva et al, 2005; Westbrook et al, 2005). Thus, genetic screens offer a strategy to discover drugs or drug targets that would not compromise the health of normal cells (Hartwell et al, 1997).

In a simplistic way, we can distinguish between “positive” and “negative” selection screens where mutants become either enriched or depleted under certain conditions. Positive screens are ideally applied to uncover genes that confer resistance to a given cellular perturbation, whereas negative screens assist the identification of oncogenic pathways that promote malignancy or compound-sensitive mutant strains (Ngo et al, 2006; Nijman, 2011; Schlabach et al, 2008; Smida & Nijman, 2012).

The availability of RNA interference (RNAi) and collections of RNAi resources that encompass the entire human and mouse genomes made it feasible to suppress gene expressions systematically on a large scale and to study the effects of gene suppression on specific cellular processes or signalling pathways (Brummelkamp & Bernards, 2003; Brummelkamp et al, 2002; Elbashir et al, 2001; Grimm, 2004; Iorns et al, 2007; Moffat & Sabatini, 2006). Transient or stable gene silencing by RNAi in both cultured mammalian cells and animal models has already provided insights into many cell biological processes such as induction of apoptosis or proteasome function and proven to be applicable to generate individualized treatments for cancer (Berns et al, 2004; Grueneberg et al, 2008; Kaelin, 2009; Mazurkiewicz et al, 2006; Paddison et al, 2004; Silva et al, 2005; Westbrook et al, 2005; Zuber et al, 2011). Here, it is also worth noting that recent screening efforts applying large sets of cell lines against compounds uncovered clinically relevant, conserved cancer vulnerabilities (Barretina et al, 2012; Garnett et al, 2012).

What is a tool set for genetic screening composed of? First of all, the ectopic expression of genes is achieved by collections of complementary DNAs (cDNAs). They induce a novel phenotype in a dominant fashion and can be applied in so-called gain-of-function genetic screens. Knock-down of target gene expression is carried out by either chemically synthesized small interfering RNAs (siRNAs) or

virus-based vectors encoding short hairpin RNAs (shRNAs), resulting in either temporary or stable inhibition of gene expression, respectively (Kaelin, 2009; Mazurkiewicz et al, 2006). It has become feasible to screen large-scale siRNA- or shRNA-based libraries targeting the human genome in a variety of cell types (Mazurkiewicz et al, 2006; Paddison et al, 2004; Schlabach et al, 2008; Silva et al, 2005).

Moreover, analysis of mixed populations is provided by the application of signature-tagged approaches (Mullenders & Bernards, 2009). Besides analysis of pathogenic microorganisms (Hensel et al, 1995) and yeast (Forsburg, 2001; Shoemaker et al, 1996; Winzeler et al, 1999), such massive parallel barcoding screens enable high-throughput analysis of mammalian cells (Berns et al, 2004; Muellner et al, 2011; Nijman, 2011; Paddison et al, 2004). Here, each mutant is tagged and therefore represented with a unique DNA sequence that can be amplified by PCR. Consequently, the abundance of barcodes can be quantified and deconvolved by the use of microarray analysis or sequencing (Forsburg, 2001; Kaelin, 2009; Mazurkiewicz et al, 2006; Nijman, 2011; Schlabach et al, 2008). This approach allows the detection of mutants, pre- and post-selection (Hensel et al, 1995), whereby the sequence tag acts as a molecular barcode identifier to monitor the presence of each mutant in the mixed population (Mazurkiewicz et al, 2006). Several groups have already demonstrated the feasibility and power of signature tagging in conjunction with siRNA to facilitate screening in mammalian cells in a high-throughput fashion (Berns et al, 2004; Mazurkiewicz et al, 2006; Paddison et al, 2004).

1.3.3 Aim of the study - Luminex-based combinatorial genetic and chemical screening in breast cancer cells

Strikingly, efforts for a systematical stratification of breast cancer therapy in dependency on the genetic make-up of breast tumours have not been successfully conducted thus far.

In this study, we seeked to contribute to a better stratification of breast tumours and their treatment options and described a combinatorial approach to discover synthetic lethal and drug resistant interactions relevant for breast cancer. We generated a genetically tractable human cell model mimicking the diverse genetic background of breast tumours. In this panel of isogenic cell lines we screened a large siRNA library mainly composed of clinically actionable and druggable kinases that are targets for therapeutic development, revealing interactions between frequently occurring driver mutations and potential drug targets. In parallel, we systematically explored the potential impact of a set of FDA-approved drugs and clinically relevant experimental compounds on breast cancer cell survival. Via linking the genetic aberration to treatment outcome and extensive follow-up studies, this work aims to improve personalized treatment on breast cancer patients.

Importantly, the dual approach for a combined synthetic lethal and drug resistance screen as presented in this thesis differs from other screening strategies for the identification of genetic interactions in two major aspects: First of all, our screens showed high flexibility facilitating the incorporation of siRNA- or shRNA-driven knock-down and cDNA overexpression. Second, the multiplexed fashion enabled high-throughput interrogation of thousands of gene-gene and gene-drug interactions. When compared to existing efforts, this increases the number of interactions that are screened per well by up to two orders of magnitude (Muellner et al, 2011).

1.4 PDK1 signalling: A new hope for breast cancer patients?

In breast cancer -just as in other types of cancer-, the same signalling pathway is frequently targeted by mutations. Yet, strikingly, the components of the pathway that are mutated are not always the same (Bernards, 2010). Thus, a logical starting point to identify new drug targets for breast cancer patients may be proposed by searching for novel components and modulators of cancer-relevant signalling pathways (Mullenders & Bernards, 2009).

As mentioned in section *1.1.1.2*, the phosphatidylinositol 3-kinase (PI3K) - phosphoinositide-dependent kinase 1 (PDK1) - AKT signalling cascade is one of the most frequently altered pathways in breast cancer (Curtis et al, 2012; Miller et al, 2011; Polyak, 2007; Shah et al, 2012). Indeed, mutations in the pathway components are found in more than 70% of breast cancer cases. In such patients this signalling cascade can be activated by several genetic alterations including amplification of HER2, mutation of PIK3CA, loss of PTEN, mutation of AKT and amplification or overexpression of PDK1. Aberrant activation of this pathway promotes breast cancer cell proliferation and survival and can confer resistance to some of the current breast cancer therapies such as chemotherapy, endocrine therapy and HER2-targeting (Campbell et al, 2001; Clark et al, 2002; Frogne et al, 2005; Ghayad et al, 2008; Hennessy et al, 2005; Kim et al, 2005; Liang et al, 2003; Tokunaga et al, 2006). Thus, in breast cancer patients, activation of this pathway has been associated with poor prognosis, advanced stage and histological grade. Consequently, the PI3K-PDK1-AKT signalling is considered as an attractive target for anti-cancer drug development (Ghayad & Cohen, 2010). Indeed, several tyrosine kinase inhibitors and signal transduction inhibitors targeting kinases in this pathway have been developed and many are being investigated in the clinic. Of interest, taking one step further and moving actually beyond the target, tumour cells may obtain alterations in downstream pathway components that trigger therapy resistance. This makes it of great value to survey modifications present in the entire pathway. Such an approach could help to define new pathway components as well as connections between pathways that influence patient response so that an optimal therapeutic agent can be selected (Majewski & Bernards, 2011).

Given that oncogenic pathway signatures can serve as a guide to targeted therapy and that pathway-targeted treatment indeed becomes increasingly used in cancer

(Bernards, 2010; Bild et al, 2006; Lee et al, 2007; Potti et al, 2006), it is not unfounded to predict that screening efforts with druggable gene families for their ability to modulate the PI3K-PDK1 signalling may represent a promising approach and provide potential novel avenues for the effective treatment of breast cancer patients.

1.4.1 Complexity of PI3K-PDK1 signalling cascade

Among the most identified pathways is definitely the PI3K-PDK1 signalling pathway. In the last years, we have learnt that deregulations in an oncogenic pathway underlie the oncogenic phenotypes and hence reflect the biology and clinical outcome of several cancers (Bild et al, 2006). In order to appreciate its key role in breast tumour progression and therapeutic implications, we first need to understand the complex architecture of the PI3K-PDK1 signalling, which alterations to find in breast cancer and their associations with sensitivity to therapeutics. Only a closer look on the state of clinical development of inhibitors targeting different members and/or activators of this pathway with a particular interest in PDK1, a master growth factor signalling kinase, can clearly pinpoint the unmet clinical need for an efficient and specific targeting of this essential signalling network in numerous cancer cohorts including breast cancer.

1.4.1.1 PDK1 as a major signalling hub

Growth factors, insulin, inflammatory mediators, hormones, neurotransmitters, immunoglobulins and antigens all stimulate central cellular processes like proliferation and survival by activating signalling cascades involving various protein kinases (Marone et al, 2008; Uras et al, 2012). Among these, PI3K and PDK1 represent critical components (Cantley, 2002; Uras et al, 2012).

How do we define PI3Ks? This kinase family is composed of four classes of conserved lipid kinases: three subclasses phosphorylate lipids, while the forth group covering mTOR, ATM, ATR and DNA-PK phosphorylates proteins. Kinases of class I contain heterodimers composed of a p110 catalytic subunit (p110α, p110β, p110δ) bound to one of the p85 regulatory subunits (p85α, p85β, p55α, p50α, p55γ). Besides several other crucial components of this cascade including PDK1 and its prominent

downstream target AKT, class I PI3Ks represent the most broadly studied and serve as the major targets for cancer treatment (Klement et al, 2012; Zhao & Vogt, 2008).

Just as any other signalling network, PI3K-PDK1 cascade is highly organized and tightly regulated by a complex network of positive and negative feedback loops together with crosstalk among pathways (Klement et al, 2012). The activation of PI3K-PDK1 signalling is known to be triggered by several mechanisms: it can be activated by G-protein coupled receptors, by interaction with G-proteins such as RAS or tyrosine kinases like Src that phosphorylate their regulatory subunit (Katso et al, 2001; Liu et al, 2009). Alternatively, activation of this pathway in breast cancer can be determined by aberrantly activated receptor tyrosine kinases (RTK) such as insulin-like growth factor 1 receptor (IGF1-R), fibroblast growth factor receptor (FGFR) or ErbB including HER2 (Knowlden et al, 2008; Knuefermann et al, 2003; Stern, 2008). When bound to the ligand, the intracellular portion of RTK is auto-phosphorylated and functions as a docking station for many intracellular proteins including PI3K. The activation of PI3K is then induced either directly by localization of the catalytic subunit to the membrane where it is in close proximity to its substrate, that is mainly PIP2 (Carnero, 2010), or indirectly by interaction with insulin receptor substrate-1, a receptor-associated adaptor molecule (Klement et al, 2012). Once activated at the membrane surface, PI3K produces second messenger molecules composed of phosphoinositides that prepare the protein kinase group AGC (cAMP-dependent, cGMP-dependent, protein kinase C) for activation (Uras et al, 2012).

To date, we are aware of at least twenty three members of the AGC kinases that need to be phosphorylated by PDK1 at a conserved residue in their activation loop -also known as T-loop- (Pearce et al, 2010; Peifer & Alessi, 2008; Rodriguez-Viciana et al, 1997; Toker & Cantley, 1997; Uras et al, 2012; Vanhaesebroeck et al, 1997; Williams et al, 2000). These kinases include prominent enzymes such as the proto-oncogene AKT -also known as Protein Kinase B (PKB)-, Protein Kinase C (PKC), the serum- and glucocorticoid-induced kinase (SGK), the p90 ribosomal S6 kinase (RSK) and the p70 S6 kinase (S6K) (Figure 9) (Pearce et al, 2010; Peifer & Alessi, 2008; Rodriguez-Viciana et al, 1997; Toker & Cantley, 1997; Uras et al, 2012; Vanhaesebroeck et al, 1997; Williams et al, 2000). Together, the substrates of PDK1 present a central platform for proliferative signal transduction and thus mediate diverse effects of growth factors on cell proliferation and survival (Figure 10) (Mora et al, 2004; Rodon et al, 2013; Uras et al, 2012). Indeed, they act as agonists of cell growth and translation. In particular, AKT plays a key role in inhibition of

mitochondria-mediated and Fas-L-mediated apoptosis, for instance, by inhibiting BAD or GSK3 (Brazil & Hemmings, 2001). It is involved in cell cycle regulation via inhibiting FoxO-3a or p53 (Brazil & Hemmings, 2001; Duronio, 2008). In addition, AKT is known to promote transcription and translation, for instance, by inhibiting TSC1/2 and activating NF-κB (Brazil & Hemmings, 2001; Duronio, 2008). On the other hand, SGK is implicated in regulation of ion transport, while S6K controls protein synthesis and amino acid storage. PKC-α is activated in a diacylglycerol dependent manner and in turn activates gene transcription via the IKK/NF-κB, Fos/Jun/AP1 and MAPK pathways (Weinberg, 2007), whereas RSK regulates cellular growth and proliferation via the MAPK/ERK pathway (Roux et al, 2003). Overall, the downstream effectors of PDK1 dictate cellular growth, proliferation, survival, metabolism and motility, classifying PDK1 as a major signalling hub downstream of PI3K (Downward, 2004; Engelman et al, 2006; Hanada et al, 2004; Lawlor & Alessi, 2001; Uras et al, 2012). Of interest, these kinases are frequently found altered in many diseases such as cancer (Downward, 2004; Engelman et al, 2006; Hanada et al, 2004; Lawlor & Alessi, 2001; Uras et al, 2012).

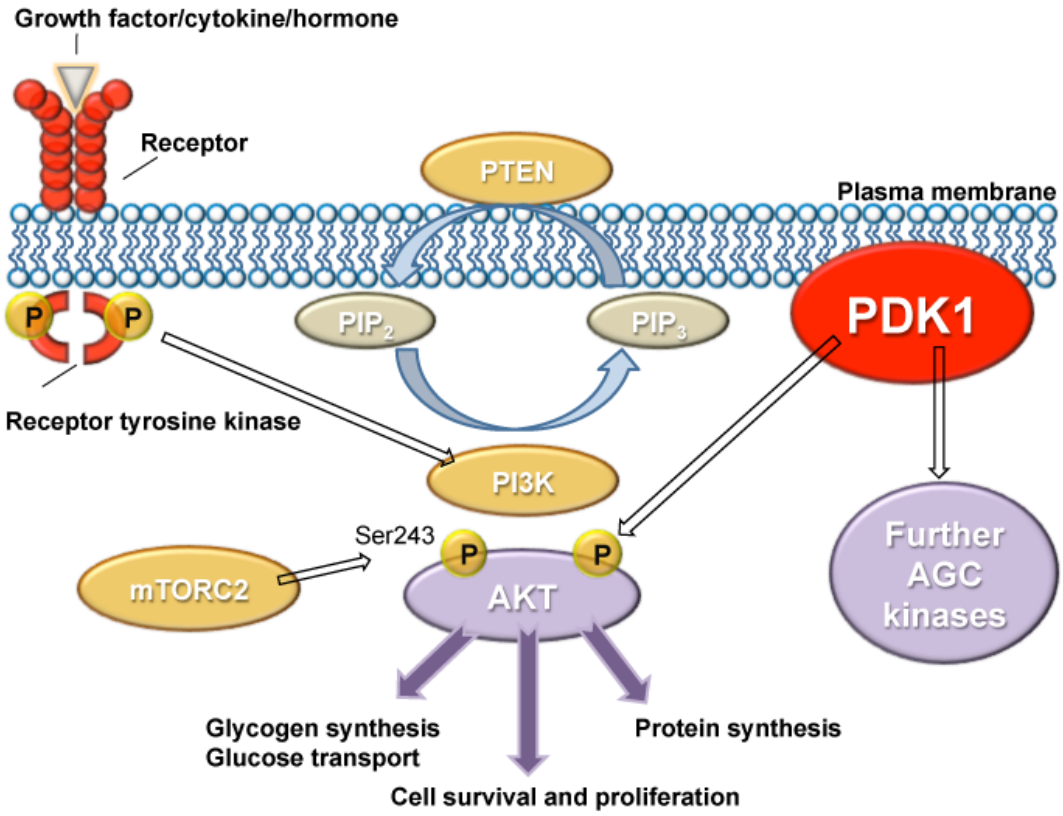

Figure 9. PDK1 signalling

PDK1 sits at the hub of receptor tyrosine kinase signalling and orchestrates numerous biological processes by phosphorylating AKT at Thr308 that is essential for activation in cells, and further AGC kinases. Once aberrantly upregulated due to excessive growth factor receptor activation and PTEN mutations, PDK1 stimulates its downstream kinases. This favours tumourigenesis and triggers tumour angiogenesis, invasiveness and progression. An additional phosphorylation of AKT at Ser473 is achieved by the mTORC2 complex and contributes to AKT activation.

PDK1 action is assigned by co-localization with substrate and by availability of target site, both characteristics that may enable it to fulfil its responsibilities in both resting and stimulated cells (Belham et al, 1999). A sobering notion is that though PDK1 sits at the hub of many diverse signalling pathways, to date the mechanisms that keep PDK1 activity in check are not comprehensively studied (Uras et al, 2012). However, we have to appreciate that these are of great importance because interfering with the activation of its downstream effectors could present enormous therapeutic value (Uras et al, 2012). Given the importance of PDK1 as a kinase, it is remarkable that it is constitutively active by *in trans* auto-phosphorylation of its T-loop residue at Ser241 (Casamayor et al, 1999; Uras et al, 2012). In fact, we believe that the regulation of substrate accessibility presents a major mechanism to control PDK1 activity (Uras et al, 2012). For AKT, both kinases PDK1 and AKT are recruited to the plasma membrane by binding to the lipid second messenger molecule PIP3

produced by PI3K via their Pleckstrin Homology (PH) domains (Uras et al, 2012). In the case of other AGC kinases such as S6K and SGK, an initial phosphorylation step on the hydrophobic motif by other kinases triggers interaction with the PDK1-interacting fragment (PIF) pocket in close proximity to the catalytic domain of PDK1 in the cytosol, thereby promoting T-loop phosphorylation in a PIP3 independent fashion (Bayascas et al, 2008; McManus et al, 2004; Pearce et al, 2010; Uras et al, 2012). Furthermore, we know that PDK1 shuttles between the nucleus and the cytoplasm in a growth factor dependent manner yet the role of this in terms of downstream activation has not been understood (Lim et al, 2003; Scheid et al, 2005; Uras et al, 2012). Given that PDK1 is a crucial regulator of numerous prominent target kinases, it is highly likely that additional regulatory mechanisms remain to be identified (Uras et al, 2012).

1.4.1.2 PDK1 signalling in disease

As outlined above, we understand that activation of PDK1 signalling cascade has a positive effect on cell growth, survival and proliferation. Hence, constitutive upregulation of PDK1 and its downstream signalling has a destructive effect on cells promoting uncontrolled proliferative capability, enhanced migration and adhesion-independent growth (Marone et al, 2008). These events favour not only the development of inflammatory and autoimmune disease but also the formation of malignant tumours (Marone et al, 2008).

In fact, genes that regulate PIP3 generation, the major product of PI3K, are frequently found mutated, amplified or silenced in somatic and germline cells (Table 9) (Klement et al, 2012; Liu et al, 2009; Rodon et al, 2013). Such mutations occur significantly in around 50% of common human cancer types including breast, lung, gastric, prostate, haematological and ovarian tumours and convey the cancerous cells with aberrantly high levels of the phospholipid PIP3 (Peifer & Alessi, 2008). This lipid second messenger leads then to hyperstimulation of PDK1, thereby facilitating constitutive activation of AGC kinases including AKT and S6K. This precise regulation by PDK1 influences angiogenesis, promotes cancer cell proliferation and reduces apoptosis rates (Peifer & Alessi, 2008). Contrarily, is PDK1 absent, the PDK1-driven downstream signalling of AKT, S6K and RSK fail to be stimulated by growth factors, with complete inhibition of phosphorylation on the threonine residue in their activation loops (Alessi et al, 1997; Williams et al, 2000). In addition, how PDK1 contributes to the oncogenic signal is presented by the activating E17K

mutation in the PH domain of AKT, which enables AKT phosphorylation by PDK1 even when of extracellular stimuli are missing and at low PIP3 levels. Henceforth, we can think of the role of PDK1 in cancer as a constitutively active and essential activator of the oncoprotein AKT, which holds the promise to represent a limiting factor in proliferative signal transduction.

Furthermore, PDK1 overexpression in mammary epithelial cells is known to induce transformation and adhesion-independent growth in soft agar (Xie et al, 2003). As a consequence, PDK1 is found overexpressed and amplified in 20% of human breast carcinomas (Table 9) (Brugge et al, 2007; Liu et al, 2009; Rodon et al, 2013). Moreover, recent studies have shown that breast cancer cells expressing low AKT levels depend on the PDK1 target SGK for cell proliferation (Vasudevan et al, 2009).

Genetic alteration	**Cancer type**	**Frequency %**
p110α (PIK3CA)		
Mutations	Breast	25
	Endometrial	26
	Urinary tract	21
	Colon	12
	Ovarian	10
Amplifications	Lung (squamous cell carcinoma)	66
	Lung (adenocarcinoma)	12.5
	Head and neck	42
	Gastric	37.9
	Colon	9
	Breast	
p110β (PIK3CB)		
Amplifications	Ovarian	5
	Breast	5
Increase in activity and expression	Colon	70
	Bladder	89
PDK1		
Amplifications and overexpression	Breast	20
AKT		
AKT1 mutation (E17K)	Breast	4
	Colon	1
	Ovarian	1
	Endometrial	NA
AKT1 amplifications	Gastric	20

	Breast	1
AKT2 mutation	Colon	1
AKT2 amplifications	Ovarian	14.1
	Pancreas	20
	Head and neck	30
	Breast	3
AKT3 mutation (E17K)	Skin	1.5
AKT3 amplifications	Glioblastoma	2
	Breast	9.9
PTEN		
Monoallelic loss	Glioblastoma	75
	Colon	20
	Breast	40-50
	Lung	37
	Prostate	42
	Gastric	47
Biallelic mutations	Endometrial	50
	Glioblastoma	30
	Prostate	10
	Breast	5
	Colorectal	7
Loss of expression *	Endometrial	NA
	Prostate	NA
	Breast	NA
	Ovarian	NA
	Glioblastoma	NA
	Melanoma	NA
Other regulators of PI3K		
Loss of expression of INPP4B	Prostate	NA
	Breast (triple-negative)	NA
	Ovarian	NA
Mutation of *PIK3R1*	Glioma	4
	Colon	3
	Ovarian	<5

Table 9. Molecular alterations in the PI3K pathway underlying human cancer

Adapted from (Liu et al, 2009; Rodon et al, 2013). * Loss of expression of PTEN can be caused by mutation, loss of heterozygosity or epigenetic factors (i.e. promoter hypermethylation or altered expression of microRNAs (miR-21 and others)). INPP4B, type II inositol 3,4-bisphosphate 4-phosphatase; NA, not available; PDK1, 3-phosphoinositide-dependent kinase 1; PI3K, phosphoinositide 3-kinase; PIK3CA, PI3K catalytic subunit α-isoform; PIK3CB, PI3K catalytic subunit β-isoform; PIK3R1, PI3K regulatory subunit-α; PTEN, phosphate and tensin homologue

Moreover, *PDK1*-deficiency causes pancreatic hypoplasia in mice when knocked out in pancreatic cells and cardiac arrest by sensitizing cardiomyocytes to hypoxia (Mora et al, 2003; Westmoreland et al, 2009). It is also worth noting that *PDK1*-deficiency in mice results in embryonic lethality due to numerous developmental abnormalities in brain formation (Lawlor et al, 2002). In addition, mice with hypomorphic PDK1, that causes a reduction of PDK1 levels by 90%, are viable yet 40-50% smaller than wild type mice, which is associated with decreased cell volume in affected mice rather than cell number (Lawlor et al, 2002).

Of interest, the significance of the PDK1 signalling in carcinogenesis has also been spotlighted by the reports that the activity of PTEN (phosphatase and tensin homologue deleted on chromosome 10), a dual lipid and protein phosphatase that converts PIP3 to PIP2, is dampened by mutations, deletions or promoter methylation silencing at high frequency in many primary tumours and metastases (Carnero, 2010). Interestingly, PTEN-deficient glioblastomas exhibit elevated levels of PIP3, AKT and S6K activity and importantly, their proliferation can be blocked by PDK1 depletion (Flynn et al, 2000). Moreover, $PTEN^{-/-}$ cell progenitors depend on PDK1 for their ability to transform or develop into invasive and fatal T lymphomas, further supporting the role of PDK1 as a limiting factor (Finlay et al, 2009). Furthermore, heterozygous $PTEN^{+/-}$ mice develop a wide range of tumours. Of interest, these animals can be protected from tumour formation when PDK1 expression is reduced. Hence, this model successfully mirrors the therapeutic power of PDK1 inhibitors (Peifer & Alessi, 2008).

1.4.1.3 Targeting PDK1 signalling by small molecule inhibitors

As introduced above, in multiple cancers, PDK1 is overexpressed and/or its signalling is upregulated promoting tumour invasiveness, angiogenesis and progression (Liu et al, 2009; Peifer & Alessi, 2008). Especially, PI3Ks, PDK1, AKT and mammalian target of rapamycin (mTOR) are favourite targets for therapy (Figure

10, Figure 11) (Garcia-Echeverria & Sellers, 2008; Liu et al, 2009; Rodon et al, 2013). The reasoning behind this is that they are readily accesible drug targets a priori and according to the addiction hypothesis, tumour cells with the activated pathway will be highly dependent on its activity to survive (Carnero, 2010). Henceforth, inhibition of PDK1 by small molecules is predicted to result in effective inhibition of cancer cell proliferation and therefore be therapeutically beneficial. Indeed, biological characterizations on cancer cell growth *in vitro* and *in vivo* have advanced PDK1 as a valid drug target for clinically effective small molecule agents to treat tumours with elevated AKT and S6K activity (Peifer & Alessi, 2008). Moreover, several X-ray crystal structures of PDK1-ligand complexes are available, promising a solid basis for structure-based inhibitor design. Together, the scientific value and therapeutic potential of PDK1 inhibitors are certainly evident. For instance, upon RNAi-mediated knock-down of PDK1, human breast cancer cell line MCF7 displays increased sensitivity to tamoxifen and to ICI 182780 (Fulvestrant) *in vitro*, a drug that yields ER degradation (Iorns et al, 2009). Moreover, compared to cells with AKT overexpression, mouse mammary cells with elevated levels of PDK1 are rendered resistant to gemcitabine. In line with this, high levels of phosphorylated PDK1 rather than phosphorylated AKT confer enhanced resistance to gemcitabine exposure in human breast cancer cells (Liang et al, 2006). In addition, in PTEN defective glioblastoma cells, PDK1 depletion decreases levels of activated AKT and subsequently inhibits cell proliferation *in vitro* (Flynn et al, 2000). Moreover, the fact that the allosteric AKT inhibitors, Akti-1 and Akti-2, undergoing clinical trials, that rely on inhibition of the phosphorylation of AKT by PDK1, also advances PDK1 as a master regulator and significant target (Barnett et al, 2005; Green et al, 2008). Therefore, we can hypothesize that PDK1 may represent a superior inhibition point to AKT for sensitizing cells to exposure with cytostatic drugs such as gemcitabine as mentioned above. In addition, as opposed to a direct targeting of AKT, attacking PDK1 and hence indirectly repressing AKT activity may also faciliate to dampen selective pressure on tumours and thus diminish resistance in transformed cells harbouring oncogenic AKT. Of value, the knowledge that mice with hypomorphic PDK1, that causes a reduction of PDK1 levels by 90%, are smaller but viable indicates that PDK1 inhibition could result in fewer side effects and less toxicity (Garcia-Echeverria & Sellers, 2008).

Together, based on the strong biological evidence of PDK1 being a valuable target in anti-cancer drug development, the available structural data for drug design and medicinal chemistry efforts, few potent and selective compounds directly attacking

PDK1 are documented in patents and papers (Peifer & Alessi, 2008). Most of these PDK1 inhibitors are ATP competitive and thus target ATP binding site of PDK1. To date, we are aware of 11 small molecule inhibitor-PDK1 complexes in the Protein Data Bank enhancing our structural knowledge on ligand-protein interplay (Peifer & Alessi, 2008). Among different classes of structurally diverse PDK1 inhibitors are azaindoles, indazoles, indenylpyrazoles, tetracyclics and tricyclics (Xu et al, 2009). For instance, UCN-01 is a staurosporine derivative kinase inhibitor that is effective on PDK1 activity with an IC50=5nM (Komander et al, 2003). Though phase I clinical trials are encouraging, UCN-01 is a non-specific kinase inhibitor which has the disadvantage of being prone to off-target effects (Garcia-Echeverria & Sellers, 2008). Moreover, two ATP competitive binders that impair PDK1 activity are in clinical trials, BX320 and BX517, and display better selectivity profile than UCN-01 (Garcia-Echeverria & Sellers, 2008). In addition, celecoxib that prevents AKT activation in various cancer types via inhibition of PDK1 kinase activity with IC50=30μM is currently tested in phase II/III trials as a monotherapy or in combination (Arico et al, 2002; Garcia-Echeverria & Sellers, 2008). Furthermore, a derivative of celecoxib based on optimized structural design, OSU-03012, results in slightly improved anti-proliferative effects (Zhu et al, 2004). In addition, PHT-427 is a PH domain inhibitor. Interestingly, this compound was initially generated to interact with the PH domain of AKT. Yet, it also blocks the PH domain of PDK1 and in turn reduces xenograft size with minimal toxicity (Meuillet et al, 2010).

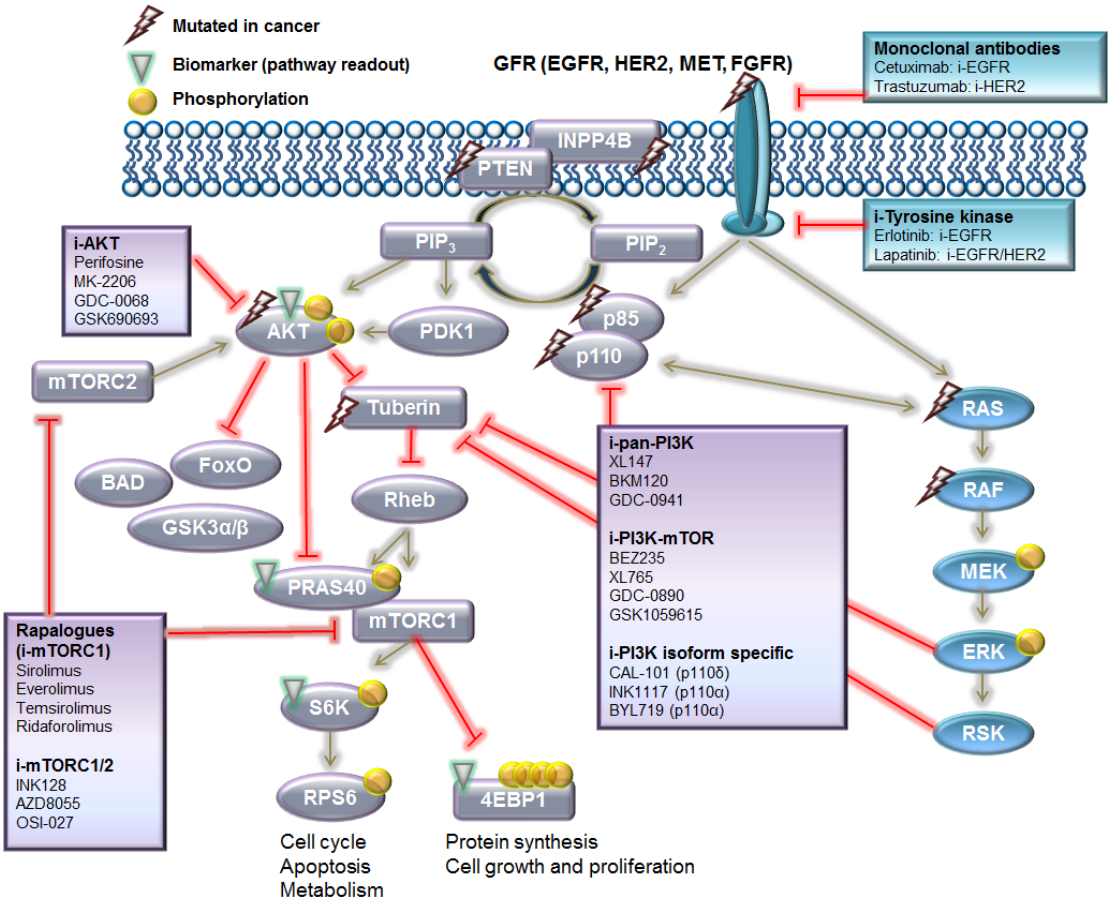

Figure 10. PI3K-PDK1 signal transduction and its targeting nodes in cancer with drugs in use or clinical development

The phospholipid PIP3 produced by the heterodimerized p110 and p85 PI3Ks promotes activation of numerous kinases including PDK1 that in turn activates AKT. This prominent protein kinase triggers glycolysis through GSK3β, stimulates survival via BAD and transcription of the anti-apoptotic genes *BIM* and *FASLG* via FoxO. AKT also activates cyclin D1, cyclin E1 and the transcription factors JUN and MYC, thus regulates the cyclin-dependent kinase inhibitors CDKN1A and CDKN1B and in turn keeps cell cycle progression in check. Metabolism, protein synthesis, cell growth and proliferation are controlled by mTORC1 via S6K and 4EBP1 axis. Angiogenesis is mediated by the mTORC1/HIF-1α/VEGF axis. PTEN and INPP4B are the prime negative regulators of the PI3K-PDK1 signalling. A broad spectrum of small molecules has been developed to directly attack individual nodes in the PI3K/PDK1/AKT/mTOR signalling cascade. Based on selectivity profiles, PI3K targeting agents fall into three groups: pan-PI3K inhibitors, dual pan-PI3K–mTOR inhibitors and PI3K-isoform-specific inhibitors. mTOR inhibitors cover allosteric inhibitors -known as rapalogues- and catalytic inhibitors. Also compounds targeting AKT are divided in both allosteric inhibitors and AKT catalytic inhibitors. 4EBP1, eukaryotic translation initiation factor 4E-binding protein 1; BAD, BCL2 antagonist of cell death; CDKN1, cyclin-dependent kinase inhibitor 1; FASLG, Fas antigen ligand; FoxO, forkhead box O; GFR, growth factor receptor; GSK3, glycogen synthase kinase-3; HIF-1, hypoxia-inducible factor 1; i-, inhibitor; INPP4B, type II inositol 3,4-bisphosphate 4-phosphatase; mTORC, mTOR complex; PDK1, 3-phosphoinositide-dependent protein kinase 1; PIP2, phosphatidylinositol (4,5)-biphosphate; PIP3, phosphatidylinositol (3,4,5)-triphosphate; PRAS40, proline-rich AKT1 substrate 1; PTEN, phosphatase and tensin homologue; RPS6, 40S ribosomal protein S6; RSK, 90 kDa ribosomal protein S6 kinase

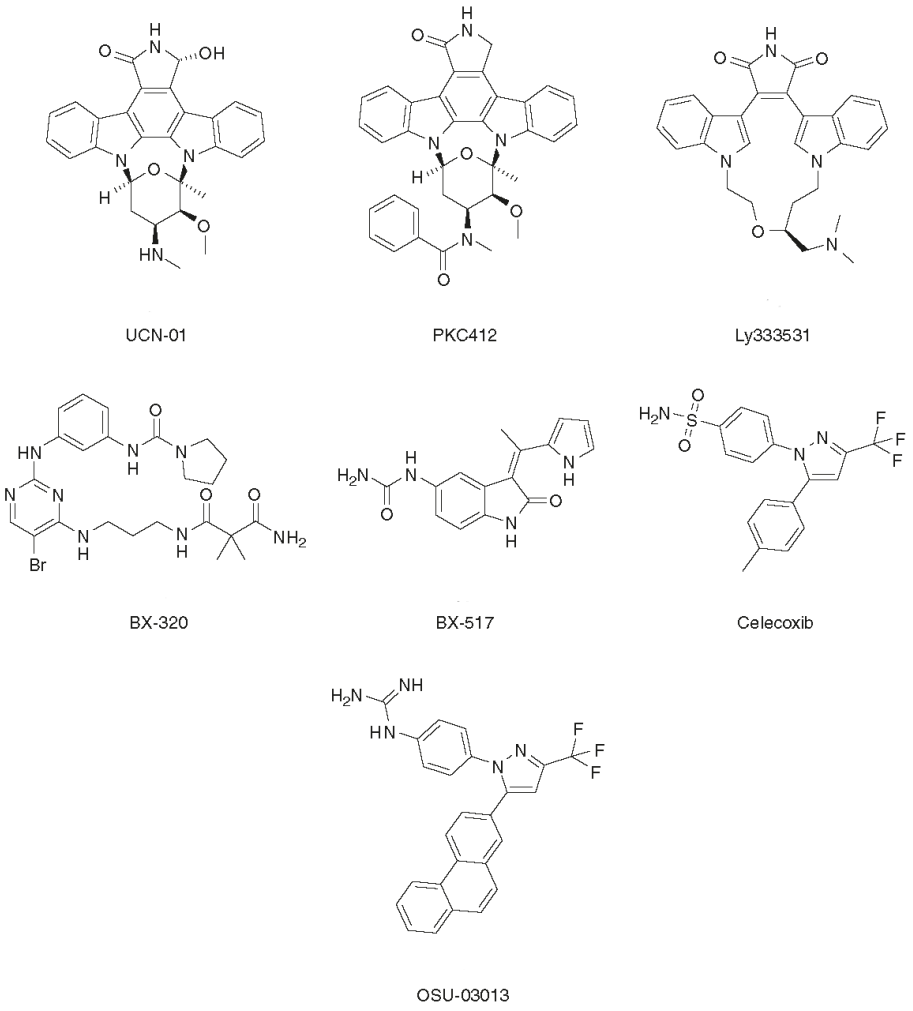

Figure 11. Representative small molecule inhibitors of PDK1

Adapted from (Garcia-Echeverria & Sellers, 2008) **Confirmation Number: 11107113 Citation Information Order Detail ID:** 63848787 **Oncogene by NATURE PUBLISHING GROUP. Reproduced with permission of NATURE PUBLISHING GROUP in the format Republish in a thesis/dissertation via Copyright Clearance Center.**

However, a sobering caveat of inhibiting the PDK1 signalling cascade that we need to keep in mind is that it can lead to insulin resistance in normal cells, that is insensitivity to insulin signals, and as a consequence to weight loss, increased blood sugar levels and type 2 diabetes (Garcia-Echeverria & Sellers, 2008). It is also highly worth noting that at the molecular level, the constitutive PDK1 auto-phosphorylation may cause a stiff conformation of the ATP binding pocket (Peifer & Alessi, 2008). This notion pinpoints that compounds with a rigid bioactive conformation eminently bind to this site. In agreement with this is that the majority of documented PDK1 targeting small molecules frequently display weak physicochemical properties due to their structurally inflexible scaffold, making it extremely challenging to translate their

potent enzymatic inhibition into cellular or *in vivo* settings (Peifer & Alessi, 2008). In addition, the stiff central pharmacophore that binds to the hinge region can often interact with other kinases, dampening selectivity profiles of the compounds (Peifer & Alessi, 2008). Altogether, these critical points signify the unmet clinical need for highly effective and specific PDK1 inhibitors. Therefore, we believe that it is extremely important to move beyond the target and to survey modifications that can interfere with PDK1 itself and/or its signalling. Such an approach could facilitate to unravel new pathway components and identification of novel, unanticipated modulators could directly categorize any validated hit as a potential target for drug design. In addition, it could also help to connect the dots between pathways that influence patient response so that an optimal therapeutic agent can be selected and applied.

1.4.2 The ubiquitin system and deubiquitinating enzymes

Over the past decades, we have learnt that upon post-translational modifications of proteins, cells respond dynamically to intra- and extracellular stimuli to control cellular processes (Haglund & Dikic, 2008; Seet et al, 2006). Among all possible polypeptide modifiers, by far the most prevalent one is the small molecule ubiquitin. This is mainly because ubiquitination frequently occurs and is crucial for the inducible and reversible control of signalling pathways that keep cellular homeostasis in check. In fact, to date, ubiquitination ranks together with phosphorylation as one of the best-studied post-translational modifications of proteins with regulatory roles in essentially all biological aspects.

Ubiquitin is a highly conserved 76-amino acid (8.5 kDa) protein present in all eukaryotes. Interestingly, under normal conditions, up to 20% of all yeast proteins are found to be ubiquitin-conjugated. Once processed from its precursor polypeptide by specific proteases, the active monomer is ready to covalently attach to other proteins through a complex, specific and tightly regulated set of processes known as ubiquitylation or ubiquitination (Fang & Weissman, 2004). The linkage of ubiquitin molecules to their target proteins occurs in three-steps through the sequential action of the ubiquitin-activating E1, conjugating E2 and ligase E3 enzymes. E1 harbours an active-site cysteine to which the carboxy-terminal glycine of ubiquitin becomes conjugated via a reactive thioester bond. Next, the ubiquitin is transferred to a cysteine residue on an E2 enzyme by a similar reaction. In the third step, the E2

interacts with a substrate-bound E3 ligase, which then catalyzes the attachment of ubiquitin to a distinct lysine in the target protein (Figure 12) (Cohen & Tcherpakov, 2010; Walczak et al, 2012). This last step occurs either directly as is the case with the RING E3 ligases, or indirectly whereby the ubiquitin is first transferred to a cysteine residue on the E3 ligase and subsequently linked to the substrate as seen with the HECT family of E3 ligases. Although rare, an additional enzyme -so-called E4- may also be required to form a polyubiquitin chain.

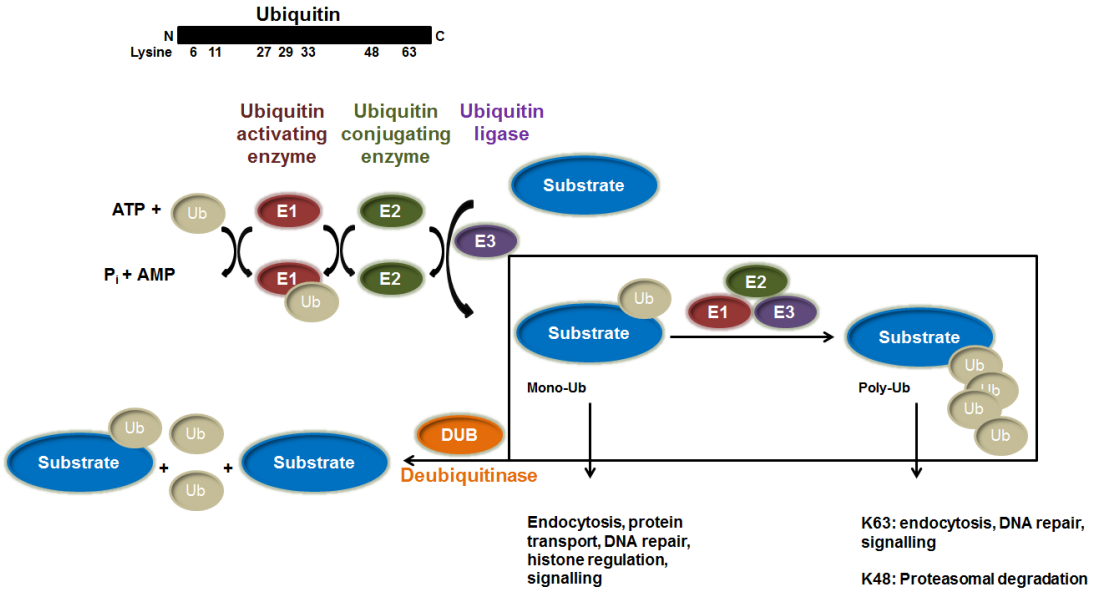

Figure 12. Key steps in protein ubiquitination

Ubiquitin has seven lysine residues. The conjugation of ubiquitin molecule to its substrate occurs in a three-step reaction via ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2 and ubiquitin ligase E3. Ubiquitin can be cleaved by deubiquitinases. Ub; ubiquitin

Of interest, there are many parallels that can be drawn between protein ubiquitination and phosphorylation: similar to phosphorylation, ubiquitin can be covalently conjugated to just one or numerous amino acid residues on the very same target protein. In fact, tagging of proteins with ubiquitin happens in multiple ways, making it a very diverse modification with distinct cellular functions. The simplest form is when a single ubiquitin moiety is attached to a single lysine residue in a substrate, a process known as mono-ubiquitination. Alternatively, when several single ubiquitin molecules are attached to several different lysines, this reaction is referred to as multiple mono-ubiquitination or multiubiquitination. However, as opposed to phosphorylation, ubiquitin can also form polyubiquitin chains. In fact, seven of its amino acids are lysines (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63) which

can themselves be targeted by ubiquitination, giving rise to chains with different linkages or branches depending on which lysine residue acts as the acceptor site for the incoming ubiquitin (Figure 12) (Walczak et al, 2012). An exception to this is the generation of linear ubiquitin chains. Here, the attachment occurs between the carboxy-terminal gylcine of the incoming and the amino-terminal methionine residue of the pioneer ubiquitin molecule (Walczak et al, 2012).

Even though ubiquitination plays a central role in degradation of proteins through proteasomal targeting and by direct sorting to the lysosome, it is now becoming clear that selective mono-ubiquitination or alternative ubiquitin chains can also regulate protein activity and are indeed necessary for proper functioning of signal transduction (Aguilar & Wendland, 2003; Bhoj & Chen, 2009; Chen & Sun, 2009; Hershko & Ciechanover, 1998; Mukhopadhyay & Riezman, 2007; Pickart, 2001; Raiborg & Stenmark, 2009; Sacco et al, 2010; Uras et al, 2012; Walczak et al, 2012). In fact, similar to phosphorylation, ubiquitination operates as a signalling agent in cellular signalling networks. Explicitly, mono-ubiquitination has become a major signalling event, mediating DNA repair, endocytosis of plasma membrane proteins, sorting of proteins into the multivesicular body, budding of retroviruses, histone activity and transcriptional regulation (Haglund et al, 2003; Haglund & Dikic, 2008; Hicke, 2001; Hicke & Dunn, 2003; Hoege et al, 2002; Katzmann et al, 2002). In addition, multiple mono-ubiquitination is thought to play a role in endocytosis of receptor tyrosine kinases and in nuclear export of p53 (Li et al, 2003; Marmor & Yarden, 2004). Moreover, in the case of polyubiquitin chains, the linkages via lysine 48 and 63 are the best characterized. In fact, Lys48-linked polyubiquitination is referred to as proteolytic signal wherein such substrates are recognized by the 26S proteasome via its ubiquitin-binding subunits and are marked for proteasomal degradation. On the other hand, Lys63-linked polyubiquitin chains are implicated in regulation of endocytosis, DNA repair and activation of NF-κB (Galan & Haguenauer-Tsapis, 1997; Haglund & Dikic, 2005; Haglund & Dikic, 2008; Hoege et al, 2002; Huang et al, 2006; Krappmann & Scheidereit, 2005). Hence, mono-ubiquitination and chains linked via lysine 63 are well characterized as significant operators in numerous non-proteolytic cellular processes to control complex signalling networks (Haglund & Dikic, 2008).

Importantly, ubiquitination is an inducible reaction promoted by signals such as extracellular stimuli, phosphorylation as well as DNA damage (Haglund & Dikic, 2005; Haglund & Dikic, 2008). This is in line with the fact that E3 ligases are

precisely regulated by signal-induced mechanisms including post-translational modifications, compartmentalization, degradation and oligomerization (d'Azzo et al, 2005; Gao & Karin, 2005; Haglund & Dikic, 2008). Moreover, just as phosphorylation, ubiquitination is a dynamic and reversible modification driven by deubiquitinating enzymes or deubiquitinases (DUBs) that cleave the isopeptide bond at the carboxy terminus of ubiquitin (Figure 12) (Nijman et al, 2005). Consequently, DUBs are able to rescue proteins from proteasomal degradation, revert conformational changes which have been induced by ubiquitination, or re-directs proteins to sites of activity from which they have sequestered (Hussain et al, 2009). The human genome encodes approximately one hundred DUBs which are cysteine- or metallo-proteases falling into five separate gene families: ubiquitin-specific proteases (USP), ubiquitin C-terminal hydrolases (UCH), ovarian tumour proteases (OTU) and Machado-Joseph disease protein domain proteases (MJD) are cysteine proteases, whereas JAMM motif proteases (JAMM/MPN domain family) consist of metalloproteinases (Figure 13) (Nijman et al, 2005; Todi & Paulson, 2011). While USPs are the most abundant with highly conserved motifs called cysteine and histidine boxes in their catalytic domain, the other four classes are thought to rescue ubiquitin falsely attached to intracellular molecules like glutathione or polyamines and to cleave newly synthesized ubiquitin polymer precursors into single ubiquitin molecules.

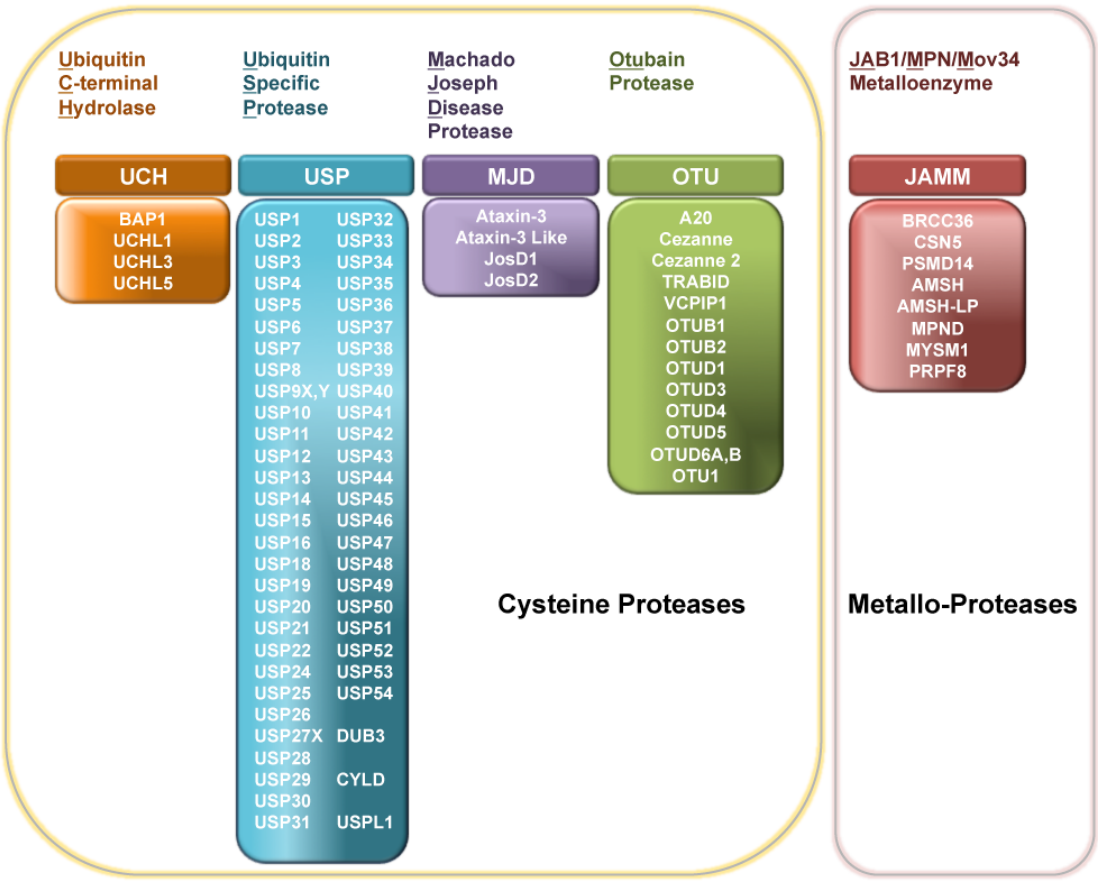

Figure 13. The five deubiquitinase families in humans

Deubiquitinases are divided into five classes depending on homology in their catalytic domain: UCH, USP, MJD, OTU and JAMM. The UCH, USP, MJD and OTU categories are cysteine proteases. The JAMM group belongs to zinc-dependent metalloproteases. Some deubiquitinases favour certain Ub-Ub linkages or a distinct Ub chain length. Ub; ubiquitin

It is also worth noting that the number of DUBs encoded by the human genome is comparable to the number of protein phosphatases, nevertheless the total number of E1, E2 and E3 enzymes exceed the number of protein kinases (Cohen & Tcherpakov, 2010).

Of interest, how DUBs are regulated is only beginning to be understood and attracts increasing interest as DUBs serve to switch off the ubiquitin signal or to stimulate a shift between different modifications of the same lysine residue (Haglund & Dikic, 2008). To date, what is known about the regulation of DUB activity? In general terms, we can say that deubiquitinases are generated as active enzymes unlike some proteases that are frequently translated as inactive precursors and get only activated by post-translational modification or through changes in pH (Nijman et al, 2005). However, structural data has also suggested that some DUBs acquire an active

conformation only upon ubiquitin-binding. In addition, some need to form complexes with other proteins in order to become active, while others are inhibited by phosphorylation or degradation (Haglund & Dikic, 2008; Nijman et al, 2005). Moreover, there is an interesting interplay between DUBs and E3 ubiquitin ligases. Indeed, interactions between DUBs and E3s have been demonstrated to regulate the stability of E3 ligases, which undergo auto-ubiquitination. Importantly, such an interaction steers the DUB to its substrate and regulates the target stability (Haglund & Dikic, 2008; Nijman et al, 2005).

1.4.2.1 Emerging roles of deubiquitinases in cancer

There is increasing evidence that deregulation of components of the ubiquitination machinery can lead to cancer development (Fang et al, 2003; Hoeller et al, 2006; Jiang & Beaudet, 2004; Nakayama & Nakayama, 2006; Singhal et al, 2008). In general terms, similar to E3 ubiquitin ligases, DUBs are both ascribed to oncogenic and tumour suppressive functions (Hussain et al, 2009; Nakayama & Nakayama, 2006). Substrates affected by alterations in E3 and DUB activity are involved in cell cycle, DNA repair, NF-κB, RTK signalling and angiogenesis, and importantly, their levels or activity are tightly regulated by ubiquitin system. Of interest, some DUBs are known to influence the stability of potent oncogenes, whereas others may negatively regulate ubiquitin-mediated signalling (Figure 14) (Fraile et al, 2012; Goldstein, 2011; Hussain et al, 2010; Sacco et al, 2010; Schwickart et al, 2010). However, given that DUBs have multiple target proteins, it is highly challenging to prove whether an individual DUB displays a net oncogenic or tumour suppressive function *in vivo* (Sacco et al, 2010). In fact, increasing evidence suggests that function varies between tissue types and stage of malignancy.

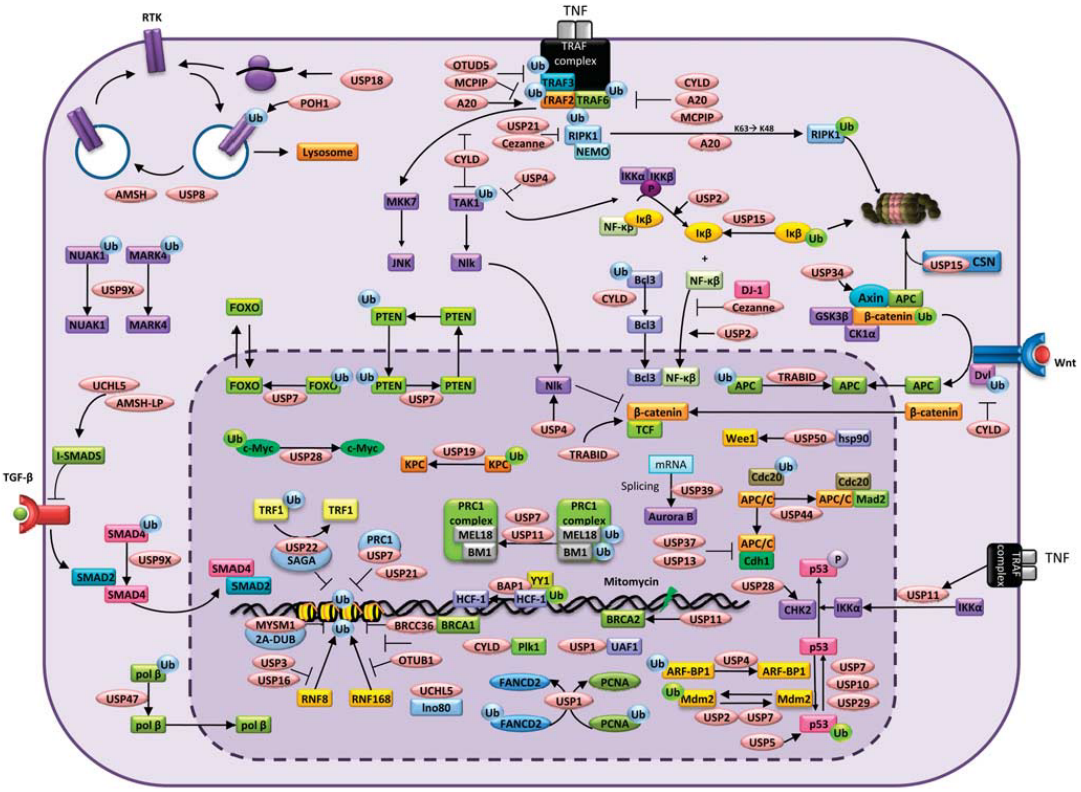

Figure 14. Deubiquitinases in cancer

Taken from (Fraile et al, 2012). Examples of DUBs involved in distinct cellular pathways are indicated in pink. Ubiquitin in green corresponds to Lys48-linked chains targeting proteins to the proteasome. Ubiquitin in blue indicates non-Lys48-linked chains. USP7, USP11, USP13, USP19, USP37, USP39, USP44, USP50 and BAP1 are involved in cell-cycle progression. USP1, USP3, USP11, USP16, USP28, USP47, BRCC36 and OTUB1 have important roles in DNA damage repair. USP3, USP7, USP16, USP21, USP22, UCHL5, MYSM1, BAP1 and BRCC36 participate in chromatin remodelling by deubiquitinating histones or other chromatin-related substrates. USP2, USP4, USP5, USP7, USP10 and USP29 intervene in p53 regulation. A20, Cezanne, OTUD5, CYLD, USP2, USP4, USP11, USP15 and USP21 participate in NF-κB signalling. USP8, USP18, AMSH and POH1 interfere in receptor tyrosine kinase trafficking. USP4, USP15, USP34 and TRABID are associated with Wnt signalling. USP9X, AMSH-LP and UCHL5 are involved in the regulation of the TGF-β pathway.

1.4.2.2 Targeting ubiquitin system and DUB inhibitors

Due to its common alteration in cancer-associated pathways and its key role in regulation of stability, localization and activity of numerous proteins, targeting the ubiquitin system emerges as a promising approach in cancer drug development

(Table 10) (Cohen & Tcherpakov, 2010). A sobering caveat is, however, reflected by the major challenge in the generation of drugs that specifically and directly target the desired ubiquitin network component or substrate with no effects on other pathways. In the recent years, several possibilities have been proposed including inhibition of ubiquitin activation or conjugation, interfering with ubiquitin ligase activity of oncogenic E3s by blocking E2 or substrate binding, or inhibition of degradation of tumour suppressors (Haglund & Dikic, 2008; Nalepa et al, 2006). Even though ubiquitin activation and proteasomal degradation steps represent therapeutically most attractive points due to the involvement of ATP-dependent and proteolytic enzymes, respectively, the major concern here is their wide action on diverse substrates and cellular pathways which may cause severe to deadly side effects (Nalepa et al, 2006). Thus, a more selective approach is proposed by intervening in the E3-substrate interaction, which is believed to lead to a more effective treatment and fewer side effects.

Inhibitors of proteasome

Company	Inhibitor	Development Stage	Disease
Millenium/Takeda	Bortezomib/Velcade	Approved	Multiple myeloma and mantle cell lymphoma
Millenium/Takeda	MLN9708	Phase I	Multiple myeloma and other cancers
ONYX (Proteolix)	Carfilzomib/PR171	Phase III	Multiple myeloma and other cancers
ONYX (Proteolix)	Onx 0912/PR047	Phase I	Multiple myeloma and other cancers
Cephalon	CEP18770	Phase I	Multiple myeloma and other cancers
Nereus Pharmaceuticals	Salinosporamid A/ NPI0052	Phase I	Multiple myeloma and leukemia

Inhibitors of E1-activating enzymes and E3 ubiquitin ligases

Company	Inhibitor	Target	Stage	Disease
Millenium/Takeda	MLN4924	NAE-E1 [b]	Phase II	Multiple myeloma and Hodgkin´s lymphoma
Roche	Nutlin/R7112	E3-Hdm2	Phase I	Blood cancers and solid tumours
Johnson &	JNJ26854165	E3-IAP	Phase I	Multiple myeloma

Johnson				and solid tumours
Genetech/Roche	GDC-0152	E3-IAP	Phase I	Metastatic malignancies
Novartis	LCL-161	E3-IAP	Phase I	Solid tumours
Ascenta Therapeutics	AT-406	E3-IAP	Phase I	Solid tumours and lymphoma
Aegera Therapeutics	AEG 35156 [a]	E3-IAP	Phase II	AML and liver cancer
Aegera Therapeutics	AEG 40826	E3-IAP	Phase I	Lymphoid tumours
Tetralogics Pharma	TL 32711	E3-IAP	Phase I	Solid tumours and lymphoma
Astellas Pharma	YM155	E3-IAP	Phase II	Lung cancer

Table 10. Inhibitors of the ubiquitin system approved or in clinical trials

Adapted from (Cohen & Tcherpakov, 2010) [a] Antisense oligonucleotide; [b] The E1-activating enzmyme for neddylation

Of great value, DUBs have also become actively investigated targets for anti-cancer drugs (Nicholson et al, 2007; Uras et al, 2012). To date, screening efforts mainly take advantage of fluorescent substrates such as Ub-AMC or a sandwich of green fluorescing protein (GFP)- ubiquitin and terbium undergoing fluorescence resonance energy transfer (Sacco et al, 2010). One caveat, however, is that these approaches are not suitable for DUBs that do not process linear chains such as AMSH, and require isopeptide linked substrates (Komander et al, 2009; Sacco et al, 2010).

Importantly, first generation inhibitors with some specificity have been successfully identified in the last years. In fact, by high-throughput screening approaches, two structurally independent compounds against the SARS coronavirus DUB papain like protease (PLpro) have been uncovered, wherein one of them has been optimized to a nanomolar inhibitory range (Ghosh et al, 2009; Ratia et al, 2008; Sacco et al, 2010). Further proof of principle has been achieved by screening efforts of a panel of small molecules against UCH-L1, which demonstrated selectivity for UCH-L1 over the related UCH-L3 (Mermerian et al, 2007; Sacco et al, 2010). Indeed, an O-acyl oxime derivative of isatins targeting inhibitor against UCH-L1, a DUB linked to Parkinson's disease and cancer as it is expressed in primary lung tumours but not in normal lung tissue, has been demonstrated to selectively kill cancer cells *in vitro* (Caballero et al, 2002; Liu et al, 2002; Liu et al, 2003; Love et al, 2007; Maraganore

et al, 1999). Moreover, selective small molecules have also been reported for USP7 and USP8 (Daviet & Colland, 2008; Sacco et al, 2010). Indeed, two companies, Progenra and Hybrigenics, have developed USP7 inhibitors, P5091 and HBX 41108, respectively, whereby HBX 41108 has exhibited a sub-micromolar inhibitory range resulting in stabilization and subsequent activation of p53 which in turn leads to apoptosis and thus impairs cancer cell growth (Cohen & Tcherpakov, 2010; Colland et al, 2009; Sacco et al, 2010). In addition, inhibitors targeting USP20, a DUB for hypoxia-inducible factor 1 α (HIF-1α) leading to its stability, are in development by Progenra, and agents for USP2, USP33 and AMSH also attract increasing interest. In fact, Novartis has patented compounds against USP2, a DUB for MDM2 that is the mouse ortholog of the E3 ubiquitin ligase HDM2 of p53, as well as against UCH-L3, a DUB linked to neurodegenerative disorders such as Parkinson's disease (Cohen & Tcherpakov, 2010; Stevenson et al, 2007). Furthermore, recently, researchers identified a small molecule with the potential to inhibit DUB activity of USP9X, USP5, USP14 and UCH37, resulting in apoptosis of tumour cells (Kapuria et al, 2010). In addition, IU1, a compound specifically targeting USP14 has shown to modulate proteasome activity (Lee et al, 2010).

It is also worthy to note that just as with kinases, there are DUBs for which inhibition needs to be avoided. One such example is the A20 deubiquitinating enzyme, which upon mutation or deletion causes or predisposes patients to inflammatory and autoimmune disorders (Cohen & Tcherpakov, 2010; Musone et al, 2008; Turer et al, 2008). A similar sobering observation is made by mutations inactivating CYLD, which cause a type of skin cancer, so-called cylindromatosis (Cohen & Tcherpakov, 2010; Kovalenko et al, 2003; Trompouki et al, 2003).

Together, though relatively underexplored, DUBs provide novel targets for drug development and possibly attractive biomarkers for activation of specific pathways (Sacco et al, 2010). Yet, development of chemotherapeutical agents targeting DUBs is still in early stages and currently no DUB inhibitor has entered clinical trials. Hence, assessing the specificity of these compounds is an essential requirement. However, selectivity has so far been only demonstrated with relatively small panels of enzymes and thus assembling comprehensive panels is needed (Cohen & Tcherpakov, 2010; Sacco et al, 2010). In addition, before agonists or compounds inhibiting DUBs will make their way to clinical use, a detailed knowledge on DUB catalytic activity, regulation and substrate specificity together with large-scale mapping efforts of target proteins are urgently required.

1.4.3 Aim of the study – USP4 regulates PDK1 mono-ubiquitination

It is of great interest that the ubiquitin system controls numerous PI3K-PDK1 signalling pathway components such as AKT and PTEN (Uras et al, 2012). In the case of PTEN, ubiquitination dictates tumour progression by differentiating between degradation and protection of PTEN (Trotman et al, 2007). It is a well known that nuclear PTEN is essential for tumour suppressive function. In line with this, recent studies have proven that mono-ubiquitination mediates PTEN nuclear import, while polyubiquitination driven by the proto-oncogenic ubiquitin ligase NEDD4-1 leads to its degradation in cytoplasm (Trotman et al, 2007; Wang et al, 2007). Furthermore, very recently it has been demonstrated that AKT ubiquitination represents an important step for oncogenic AKT activation (Yang et al, 2009; Yang et al, 2010b). In fact, AKT undergoes Lys63-chain ubiquitination, which orchestrates AKT membrane localization and phosphorylation. Importantly, the cancer-associated AKT mutant exhibits an increased AKT ubiquitination, in turn accounting for AKT hyperactivation (Yang et al, 2009; Yang et al, 2010b). Interestingly, distinct E3 ubiquitin ligases are utilized by different growth factor stimuli (Chan et al, 2012). TRAF6 was found to be a direct E3 ligase for insulin-like growth factor-1 (IGF-1)-mediated AKT ubiquitination (Yang et al, 2009; Yang et al, 2010b). Strikingly, when triggered by ErbB receptors, not TRAF6 but the Skp2 SCF complex takes over as the critical E3 ligase for AKT ubiquitination and membrane recruitment in response to EGF (Chan et al, 2012). Indeed, Skp2-deficiency results in impairment of AKT activation, glycolysis and breast cancer progression. On the other hand, there is a correlation between Skp2 overexpression, AKT activation and breast cancer metastasis. In addition, high levels of Skp2 prove to be a useful biomarker for poor prognosis in HER2-positive patients and its silencing sensitizes such tumours to Herceptin treatment (Chan et al, 2012). Moreover, Lys63-polyubiquitinated AKT has recently been shown to be deubiquitinated by CYLD, preventing lung fibrosis (Lim et al, 2012). Together, these studies emphasize the importance of this post-translational modification in the proliferative PI3K-PDK1 signal transduction, revealing an exciting therapeutic avenue for pharma industry.

However, for the major signalling hub PDK1, no post-translational modification other than phosphorylation has been documented at the time of this study. Therefore, in this work we aimed to advance our current understanding of how PDK1 and its downstream signalling are regulated. Thus, we investigated the impact of

ubiquitination and deubiquitination machinery on PDK1. We further aimed to characterize this modification by identifying its site of attachment and its function using genetic and biochemical approaches. In addition, to explore the clinical relevance of such a modification in our settings we screened a total of 70 ubiquitin proteases for their ability to modulate this highly cancer-relevant signalling pathway upon cDNA overexpression. Here, novel modifiers would directly assist potential new angles for drug design (Uras et al, 2012).

2 Results

2.1 Luminex-based combinatorial genetic and chemical screening in breast cancer cells

2.1.1 Prologue

In the last decades significant improvement on breast cancer diagnosis and therapy have been achieved. Systematical stratification of breast cancer treatment based on an individual´s tumour genetic make-up, however, have not been fully established. Indeed, the translation of our knowledge into specific and effective drug response remains highly challenging. In fact, the comprehensive genetic characterization of breast cancer shows that only few cancer genes represent readily accessible drug targets. Therefore, the identification of vulnerabilities through synthetic lethality with approved or experimental drugs or druggable agents is of great value.

Henceforth, as stated in the section *1.3.3* (Aims of the study, page 62), the purpose of this work was to link breast cancer-driving mutations with patient response to a chosen therapeutic treatment. This holds the great promise to assist clinical treatment decisions, to aid drug repositioning and to provide novel application angles. This aim was addressed by establishing a high-throughput screening platform that allowed us to systematically study how druggable gene families of interest or clinically relevant compounds of choice can influence breast cancer viability, and thus therapy responsiveness.

2.1.2 Manuscript

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A chemical-genetic screen reveals a mechanism of resistance to PI3K inhibitors in cancer

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Linking the molecular aberrations of cancer to drug responses could guide treatment choice and identify new therapeutic applications. However, there has been no systematic approach for analyzing gene-drug interactions in human cells. Here we establish a multiplexed assay to study the cellular fitness of a panel of engineered isogenic cancer cells in response to a collection of drugs, enabling the systematic analysis of thousands of gene-drug interactions. Applying this approach to breast cancer revealed various synthetic-lethal interactions and drug-resistance mechanisms, some of which were known, thereby validating the method. NOTCH pathway activation, which occurs frequently in breast cancer, unexpectedly conferred resistance to phosphoinositide 3-kinase (PI3K) inhibitors, which are currently undergoing clinical trials in breast cancer patients. NOTCH1 and downstream induction of c-MYC over-rode the dependency of cells on the PI3K-mTOR pathway for proliferation. These data reveal a new mechanism of resistance to PI3K inhibitors with direct clinical implications.

Many factors contribute to the responses of patients to anticancer therapy, including pharmacogenetics, tumor microenvironment, vascularity and genetic aberrations[1–5]. Identifying the molecular mechanisms that influence response to anticancer drugs can improve therapy by allowing us to specify those individuals who will benefit most while avoiding unnecessary treatment. However, identifying robust biomarkers and functionally linking cancer genes to drug sensitivity has been challenging partly because of the heterogeneity among tumors. Nonetheless, catalogs describing the molecular changes in the major tumor types, which are currently emerging from sequencing efforts, will theoretically enable systematic studies into the molecular aberrations underpinning treatment response[4,6,7].

Another important objective of cancer research is to develop new anticancer treatments with increased specificity for cancer cells. For example, the monoclonal antibody trastuzumab directly targets HER2/Neu-positive breast cancer, and BRAF kinase inhibitors have recently shown promise in melanoma carrying *BRAF* mutations[8,9]. However, it is not often possible to directly translate known molecular aberrations of cancer cells into targeted therapies. For instance, the oncogenic transcription factor c-MYC is overexpressed in a variety of malignancies, but because it lacks critical hydrophobic pockets, it is challenging to target with small-molecule compounds[10,11]. Alternative approaches for identifying drugs that specifically target cancer cells are urgently needed.

The molecular changes that occur in cancer cells can result in their dependence on gene products that are not essential in normal cells[12–14]. Inhibition of these proteins would thus result in cell-cycle arrest or death of the cancer cell but would not affect the fitness of their normal counterparts. This notion, which is termed synthetic lethality (also known as synthetic sickness, induced essentiality or nononcogene addiction), provides a framework to identify drugs that do not target the cancer gene directly yet are specific for cells that contain the aberration. Indeed, the observation that cells containing *BRCA* mutations are hypersensitive to inhibition of the enzyme PARP has found its way into the clinic and serves as a paradigm underlying synthetic lethality–based therapy[15,16]. However, there are currently only a few cancer-relevant synthetic-lethal interactions that have been identified[17]. Thus, a systematic analysis of the effect of individual cancer genes on the cellular response to existing and experimental drugs may identify new targeted anticancer therapies that are directly relevant to the clinic.

The challenge of such a systematic approach is the large number of combinations among drugs and genes that would have to be analyzed. Yet the promise of insight into drug actions, as exemplified by similar screens in model organisms (most notably yeast), warrants development of suitable methods in human cells[18,19].

We developed a method to multiplex cellular fitness measurements of up to 100 isogenic cell lines using molecular barcodes to facilitate the quantitative assessment of functional drug-gene interactions in human cells. This method assists the systematic assessment of the impact of cancer aberrations on proliferation in response to a collection of drugs. Here we present the approach and use it to query a 70 × 87 drug-gene interaction matrix in breast cancer cells, which allowed the interrogation of over 6,000 drug-gene pairs. In addition to several previously identified drug-gene interactions, we report a new mechanism of resistance to PI3K inhibitors, which are currently in clinical trials[20]. This is particularly important given the large fraction of breast tumors with activating mutations in the PI3K pathway[21].

RESULTS

A platform for combinatorial fitness screens

The first step in building a platform to multiplex large numbers of combinations of genetic and chemical perturbations was to develop a sensitive and quantitative method using molecular barcodes to allow the identification of populations of cells carrying specific genetic modifications within a complex mixture. Molecular barcodes are short, nontranscribed stretches of DNA that, when integrated into the genomic DNA of a cell line, introduce a molecular

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beacon that can be selectively quantified by PCR. In a mixed population of cells that each contain a unique barcode, the relative number of cells containing a particular vector can therefore be determined by quantification of the barcodes. By pairing genetic modifications of cells (for example, the expression of an oncogene or knockdown of a tumor suppressor) with these barcodes, the cellular fitness upon drug treatment can be tracked in a multiplexed fashion. Thus, we first generated 100 lentiviral vectors carrying unique molecular barcodes flanked by common primer sites for efficient delivery into human cells (**Supplementary Results**, **Supplementary Fig. 1**).

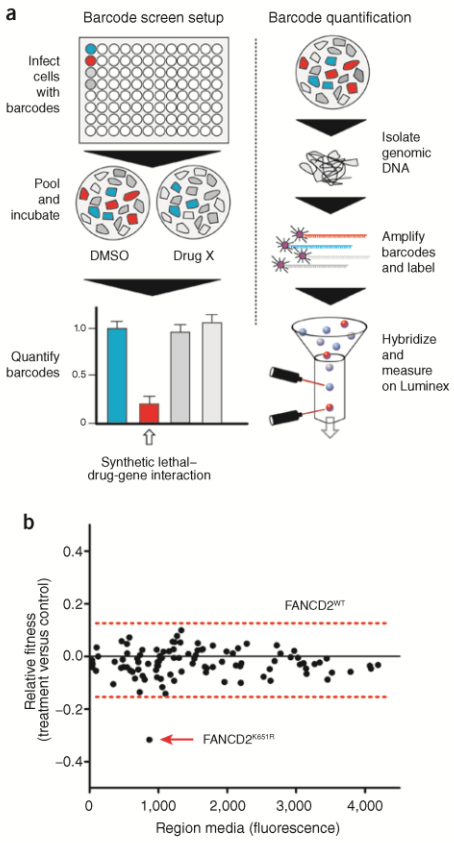

Figure 1 | Barcode screen setup, detection and performance. (**a**) Isogenic cell lines infected with a lentiviral vector carrying a unique 24-base-pair barcode sequence and a specific genetic modification (for example, cDNA or RNAi) are pooled, seeded in multiwell plates and subsequently treated with drug or DMSO control (left). The relative abundance of the barcodes in the population of cells is a proxy for the cellular fitness. In the example, the cells with the 'orange' barcode show a synthetic-lethal interaction with Drug X. After drug treatment, the pooled isogenic cell lines are harvested, genomic DNA (gDNA) is isolated and barcodes are amplified (right). Labeled product is then hybridized to Luminex microspheres, and the mixture is measured on a Luminex machine to determine the relative abundance for each of the 100 barcode sequences. (**b**) Barcoded cells expressing the inactive FANCD2^{K561R} cDNA were mixed into a pool of barcoded cells expressing wild-type (WT) FANCD2 and treated with MMC (15 ng ml^{-1}) for 5 d. Shown are the median signals for all barcodes of four independent drug treatments compared to DMSO control.

Because the quantification method is hybridization-based, we needed to exclude any cross-hybridization of barcode sequences as this could have obscured the detection of individual barcodes. For this purpose, we assembled 100 pools of barcoded vectors, each of which had a single vector omitted, and performed barcode measurements on PCR-amplified material. In all cases the absence of the correct barcode was confirmed, indicating limited cross-hybridization under these conditions (**Supplementary Fig. 5**).

Next, we determined whether the method was able to detect differences in cellular fitness in a complex mixture of barcoded cells. We used drug hypersensitivity as a benchmark as it is technically more challenging to detect the absence of a cell within a population than the increase in proliferation occurring in cells with drug resistance. Cells were infected with 1 of 95 barcoded vectors carrying a puromycin resistance gene or a barcoded vector lacking this cassette (no. 96). As expected, treatment with puromycin only killed the cells lacking the resistance gene, leaving all others unaffected (**Supplementary Fig. 6**). In addition, when all cells were pooled and subsequently treated with puromycin, a strong and highly significant (two-tailed t-test, $P < 0.0001$) depletion of the barcode associated with the vector lacking puromycin was detectable, whereas the abundance of all other barcodes remained unchanged (**Supplementary Fig. 6**). Thus, the approach was sensitive enough to detect the loss of one individual cell population within a complex mixture.

As an additional proof-of-principle experiment, we measured the known hypersensitivity of Fanconi anemia complementation group D2 (FANCD2) patient cells for the DNA cross-linking agent mitomycin C (MMC) in the multiplexed assay23. A patient-derived cell line (PD20) stably transduced with a vector expressing wild-type

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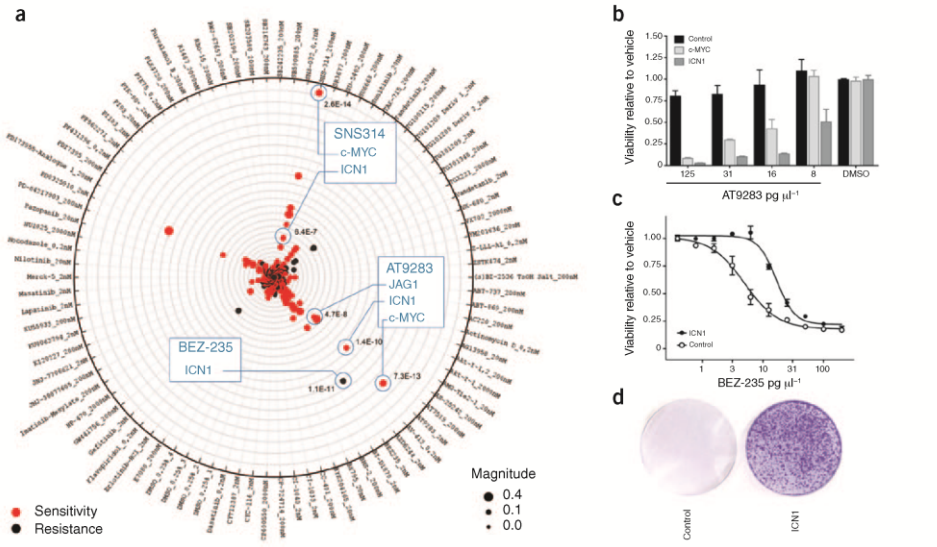

Figure 2 | Combinatorial breast cancer gene small compound screen. (**a**) Radial gene-drug interaction plot displaying the 7,743 (89 isogenic cell lines × 87 drugs) pairwise drug-gene measurements. Distance from the center indicates significance, and dot size is proportional to the magnitude of the drug versus control effect. *P* values for selected hits are indicated. (**b**) Dose-response analysis of c-MYC, ICN1 and control MCF10A cells with the Aurora kinase inhibitor AT9283. Cells were treated with the indicated concentrations for 5 d, and relative cell number was assessed. The experiment was repeated three times in triplicate. Error bars, s.d. (**c**) Dose-response analysis of ICN1 and control MCF10A cells. Cells were treated with the indicated concentrations of BEZ-235 for 5 d, and relative cell numbers were measured. The graph is based on four independent experiments in triplicate. Error bars, s.d. (**d**) Crystal violet–stained culture dishes of colony formation experiment. Cells were seeded at low density and treated with 30 pg µl^{-1} BEZ-235 for 10 d.

FANCD2 or an inactive point mutant (K561R) was infected with barcoded lentiviruses, pooled and subsequently exposed to MMC. As predicted, the barcode derived from the cells expressing the inactive mutant protein underwent depletion from the population, which could be clearly detected with our screening approach, thus confirming the MMC hypersensitivity of FANCD2 mutant cells (**Fig. 1b**, **Supplementary Fig. 7**).

Together, these experiments show that the screening platform provides a semiquantitative method to determine cellular fitness in a multiplexed format.

A synthetic-lethal and drug resistance screen

We applied our screening platform to interrogate drug-gene interactions in breast cancer cells. We first established an isogenic cell line model based on the nontumorigenic human breast epithelial cell line MCF10A. The cell line was selected because it has a relatively normal karyotype and is thought to represent a multilineage progenitor as it has transcriptional characteristics of both basal and luminal cell types[24]. Furthermore, the cells are responsive to most signaling pathways present in normal breast epithelial cells. A previously reported deletion of the *INK4A* locus and some other chromosomal aberrations could be confirmed by high-density SNP array (data not shown)[25].

We selected breast cancer–relevant genetic aberrations using an extensive literature and database search. This yielded a list of 70 genes that have been clearly linked to breast cancer, including *HER2*, *BRCA1*, *BRCA2*, *c-MYC*, *NOTCH1* and *PTEN*, which were selected for the drug-gene interaction screen (**Supplementary Fig. 8**, **Supplementary Table 1**). To mimic the aberrations of these genes in cancer, we manipulated their expression using cDNA overexpression or RNAi, and unique barcodes were introduced by lentiviral transduction to yield a total of 89 isogenic cell lines (**Supplementary Tables 1–5**). All cDNAs and the majority of knockdowns were confirmed using immunoblotting and quantitative reverse transcription (qRT)-PCR, and for a number of stable cell lines the observation of a marked morphological change indicated oncogenic transformation (**Supplementary Fig. 9**, **Supplementary Table 6** and data not shown).

After pooling of all of the barcoded cells, they were screened against a custom compound library that was selected to maximize the chance of identifying a drug-gene interaction that could be useful in a clinical setting. The library mainly consisted of clinically relevant kinase inhibitors and several tool compounds that altogether comprised 87 small molecules (**Supplementary Fig. 10**, **Supplementary Table 7**). The library was screened at various concentrations in quadruplicate, yielding over 30,000 data points (**Fig. 2a**).

Data analysis revealed several gene-drug interactions including synthetic-lethal interactions between three components of the NOTCH signaling pathway (involving JAG1, NOTCH1 and c-MYC) and the Aurora kinase drugs AT9283 and SNS-314 (**Fig. 2a**, **Supplementary Table 8**). Validation experiments with cells expressing the intracellular active domain of NOTCH1 (ICN1) or c-MYC confirmed the exquisite sensitivity to these compounds and four additional Aurora kinase inhibitors (**Fig. 2b**, **Supplementary Fig. 11**). NOTCH1 and its putative direct target gene *c-MYC* have recently been shown to have a synthetic-lethal interaction with Aurora B kinase in retinal epithelial cells, corroborating our findings and further validating the approach[26]. Furthermore, the observation

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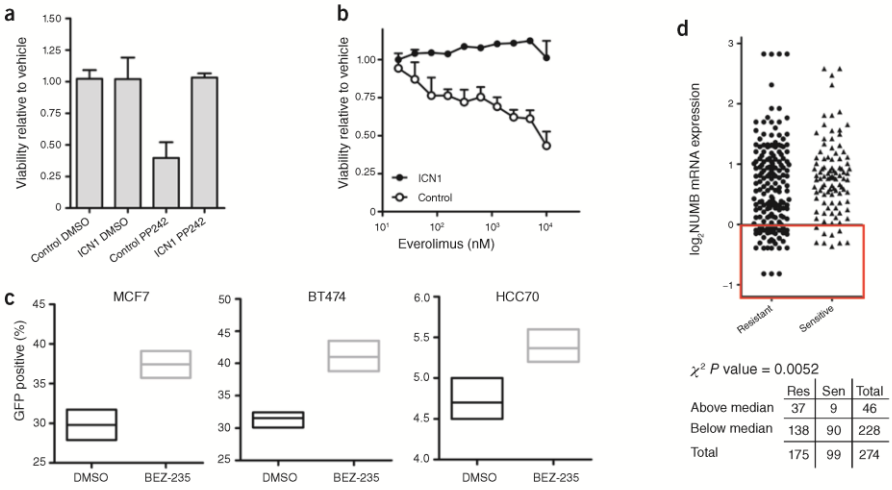

Figure 3 | NOTCH activation renders breast cancer cells resistant to PI3K-mTORC1 inhibition. (**a**) Bar graph showing relative viability of ICN1 or control MCF10A cells treated with PP242 (3.0 μM) for 5 d. Error bars, s.d. for the mean of a representative experiment performed in triplicate. (**b**) Dose-response analysis of MCF10A cells treated with the indicated concentrations of everolimus (RadO01) for 5 d. Error bars, mean ± s.d. (**c**) Box plots of GFP-positive cells transduced with an ICN1-ires-GFP virus and treated with BEZ-235 (10 pg μl^{-1}) or DMSO for 7 d. Data from three replicates are shown. (**d**) Oncomine analysis (described in Methods) of NUMB expression in 274 cell lines sensitive or resistant to PI3K-mTOR inhibitors. The red-boxed area indicates cell lines with lower-than-median expression of NUMB. Res, resistant; Sen, sensitive.

that multiple components of a single pathway cluster with two drugs targeting the same gene product illustrates how large-scale drug-gene screens in human cells could be used to elucidate drug action and gene function and is reminiscent of the synthetic-lethal screens in yeast[18,19].

NOTCH1 activation confers resistance to PI3K inhibition

Notably, our screen revealed several new drug-gene interactions. The highest-scoring resistance hit in the screen was the intracellular active domain of NOTCH1 (ICN1), which confers resistance to the dual PI3K-mTOR (mammalian target of rapamycin) inhibitor BEZ-235 (**Fig. 2a**)[27]. Given the clinical relevance of both PI3K inhibitors and NOTCH1 in breast cancer and the lack of any reported connection between the two, we decided to study this observation further[20,21].

A marked resistance to BEZ-235 in ICN1-expressing cells was observed in both short-term dose-response analysis and long-term growth assays, confirming the results from the screen (**Figs. 2c,d**; **Supplementary Figs. 12,13**). Furthermore, in cells expressing a NOTCH1 mutant that lacks the extracellular domain (NOTCH-delta E), BEZ-235 sensitivity could be restored by inhibiting γ-secretase, indicating that naturally cleaved NOTCH1 also confers resistance to PI3K-mTOR inhibition (**Supplementary Fig. 14**)[28].

Although our initial analysis revealed that ICN1 only showed a significant interaction with BEZ-235, we reasoned that ICN1 cells might also be resistant to some of the other PI3K inhibitors used in the screen. Indeed, when all remaining PI3K inhibitors were analyzed as a group, the interaction with ICN1 was also significant (one-tailed *t*-test, $P < 0.05$), an observation that indicates that the resistance could be extended to other PI3K inhibitors (**Supplementary Fig. 15**). Consistent with this result, we found that resistance to PIK90, a selective PI3K inhibitor, could be confirmed in dose-response experiments (**Supplementary Fig. 16**).

To begin to uncover the mechanism whereby activation of NOTCH1 in cells confers resistance to PI3K inhibitors, we analyzed one of the main downstream effector pathways of PI3K: the serine-threonine kinase mTOR, which is a part of the two distinct protein complexes, mTORC1 and mTORC2 (ref. 29). We found that ICN1-expressing cells were also less sensitive to PP242, an mTOR kinase inhibitor, as well as the non–ATP competitive mTOR inhibitors everolimus or rapamycin, which may affect mTORC1 more potently than mTORC2 (**Fig. 3a,b**; **Supplementary Fig. 17**)[30]. Similarly, ICN1 cells were much less affected by mTOR knockdown than control cells (**Supplementary Fig. 18**). Together, these data indicate that activation of NOTCH1 can bypass the cellular requirement for this growth pathway and that, consistent with previous reports, in these cells PI3K inhibitors mainly exert their effect by acting on the mTOR pathway[31].

Next, we investigated whether the NOTCH1-mediated resistance could also be observed in other human cancer cell lines. Notably, the breast adenocarcinoma–like cell line MCF7 and the ductal carcinoma–like cell lines BT474, HCC70 and BT549 all showed resistance to BEZ-235 treatment upon expression of ICN1 (**Fig. 3c**, **Supplementary Fig. 19**)[24]. To study whether NOTCH activation may also confer PI3K-mTOR inhibitor resistance in other tumor types, we analyzed a publicly available dataset created by GlaxoSmithKline, comprising over 300 molecularly characterized and drug-treated cell lines (described further in Methods). This revealed a significant (χ^2 test, $P < 0.01$) correlation between low expression of NUMB, a negative regulator of NOTCH, and resistance to PI3K-mTOR inhibition in cell lines derived from various tumor types, including melanoma and hepatocellular carcinoma (**Fig. 3d**)[32]. These results suggest that uncoupling proliferation from the PI3K-mTOR pathway via NOTCH1 activation may be a more general phenomenon across cancer cell lines.

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ICN1 over-rides mTORC1 signaling via c-MYC transcription

Ribosomal S6 kinase (S6K) and the eukaryotic translation initiation factor 4E–binding protein 1 (4EBP1) are the main effector molecules of mTORC1, and their phosphorylation stimulates protein translation[29]. Notably, inhibition of S6K and 4EBP1 phosphorylation was equal in ICN-expressing cells and in control cells (**Fig. 4a**, **Supplementary Fig. 21**). This suggests that ICN1 uncouples mTORC1 signaling from proliferation by a downstream mechanism.

Upon closer inspection of the screening data, we found that cells transduced with c-MYC also showed remarkable resistance to BEZ-235 and other PI3K inhibitors (**Fig. 4b**, **Supplementary Fig. 22**). Notably, the amount of c-MYC and the shift in the BEZ-235 dose-response curve were comparable to the results found for ICN-expressing cells, indicating that c-MYC may be the main transcriptional target conferring the resistance (**Fig. 4c,d**)[33–35]. In agreement with this, overexpression of the canonical NOTCH target genes *HES1*, *HEY1* or *HEY2* did not confer BEZ-235 resistance to MCF10A cells (**Supplementary Fig. 23**). Furthermore, c-MYC induction in NOTCH-deltaE expressing cells was γ-secretase sensitive, and the NOTCH3 intracellular domain—which in these cells did not induce c-MYC expression—also did not confer resistance (**Supplementary Fig. 24**).

To investigate directly if c-MYC induction was required for resistance to BEZ-235 inhibition, we inhibited c-MYC expression by RNAi in ICN1 cells (**Fig. 4e**). As predicted, knockdown of c-MYC to concentrations comparable to levels in control MCF10A cells completely reversed the resistance to BEZ-235 (**Fig. 4f**). This was not caused by a general cytotoxic effect of c-MYC knockdown as the increased sensitivity to Aurora kinase inhibitors (that is, synthetic lethality) was also reverted (**Supplementary Fig. 25**). These experiments show that c-MYC induction by ICN1 is necessary and sufficient for PI3K-mTOR resistance.

Finally, the notion that c-MYC upregulation confers resistance to PI3K-mTOR inhibition prompted us to investigate whether cell lines with c-MYC gene amplification also showed this characteristic. Indeed, c-MYC amplification was observed significantly (χ^2 test, $P < 0.01$) more often among cell lines resistant to PI3K-mTOR inhibitors (**Fig. 4g**). This effect was specific to PI3K-mTOR inhibitors as c-MYC–amplified cell lines were not resistant to Aurora kinase inhibition but rather showed a trend toward synthetic lethality, an observation that is in agreement with our previous findings (**Supplementary Fig. 26**, $P = 0.07$).

Thus, we conclude that NOTCH pathway activation uncouples PI3K-mTOR signaling from proliferation by induction of c-MYC and that this may have direct implications for patients treated with drugs targeting this pathway.

DISCUSSION

We identified a new mechanism of resistance to PI3K inhibitors in breast cancer cell lines by activating NOTCH signaling and induction of c-MYC. NOTCH activation occurs in a subset of breast cancers and is associated with tumor progression and poor prognosis, whereas *MYC* amplification is also a relatively frequent event in many other cancer types.[10,36]. PI3K- and mTOR-targeting drugs have received much attention as the PI3K-mTOR pathway is frequently 'hijacked' in a variety of malignancies, including breast cancer[21]. As tumors invariably acquire resistance to single-agent treatments, the ability to anticipate drug resistance has enormous clinical and economic value. However, mechanisms of resistance in human tumors to PI3K inhibitors have not yet been reported.

We showed that resistance occurs by the transcriptional activation of c-MYC and that this seems to uncouple regulation of translation by mTOR from proliferation. The stimulation of translation by c-MYC through the induction of eukaryotic initiation factor 4F (eIF4) family members is a known mechanism whereby c-MYC drives protein translation and is implicated in c-MYC–driven tumorigenesis[37,38]. This mechanism of how NOTCH1 activation induces resistance to PI3K inhibitors is an attractive model but remains to be confirmed. Together, these observations position activation of NOTCH or MYC as potential mechanisms of resistance to PI3K inhibitors with direct clinical implications.

We established a screening platform to systematically search for synthetic-lethal interactions and mechanisms of drug resistance in cancer cells. The ability to pair tumor genotype with cancer treatment is receiving increasing attention as the rising cost of cancer treatment is placing a burden on the healthcare system[39]. The multiplexed assay allowed the interrogation of thousands of gene-drug combinations with the potential to identify clinically relevant

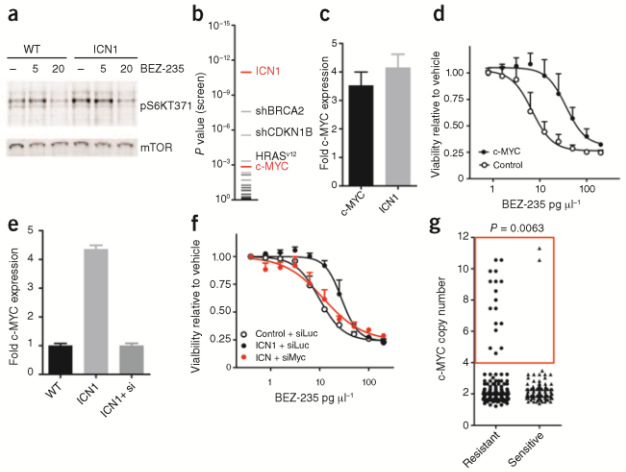

Figure 4 | c-MYC induction confers resistance to PI3K-mTOR inhibition. (**a**) Western blot analysis of ICN1 or control MCF10A cells treated with BEZ-235 (pg µl^{-1}) as indicated for 24 h. Total lysates were probed with an antibody against phosphorylated ribosomal S6 kinase (Thr371) and total mTOR as a loading control (uncropped version is in **Supplementary Fig. 20**). (**b**) Data from the screen shows c-MYC as a significant hit for resistance to BEZ-235. (**c**) Relative c-MYC mRNA concentrations in ICN1 and c-MYC cells as determined by qRT-PCR. Shown is the fold change compared to wild-type MCF10A cells. Error bars, s.d.; $n = 3$. (**d**) Dose-response curve of c-MYC or control MCF10A cells treated with BEZ-235. Cells were treated for 5 d as indicated, and relative cell number was measured. The data represent four independent experiments were performed in triplicate. Error bars, s.d. (**e**) Quantitative RT-PCR of c-MYC expression in wild-type MCF10A or ICN1 cells transfected with Luciferase siRNA and ICN1 cells transfected with c-MYC siRNA pool (ICN1 + si). Error bars, s.d. of three replicates. (**f**) Dose-response curve of cells in **e** treated with BEZ-235 or vehicle for 5 d. Error bars, s.d. of three replicate experiments. (**g**) Oncomine analysis (described in Methods) of *c-MYC* gene copy number in cell lines sensitive or resistant to PI3K-mTOR inhibitors. The red-boxed area indicates cell lines with c-MYC gene amplification (χ^2 *P* value is indicated).

interactions that could lead to new patient-stratified medicine. The method is cost effective, highly flexible and applicable to all cells transducible with lentiviral vectors, and it can be used with cDNA overexpression, RNAi or any cellular perturbation of interest.

A potential drawback of engineered cells is that they do not necessarily fully capture the tumor evolution process of primary tumor cells, and this may explain the absence of some expected 'oncogene addiction' hits in our screen. Furthermore, false negatives due to, for instance, insufficient knockdown or other technical limitations, cannot be excluded, and this may explain, for example, the absence of *PTEN* as a hit for resistance to PI3K inhibitors in our screen[40]. Nonetheless, the identification of resistance mechanisms and synthetic-lethal interactions that are conserved across many cell lines justifies the approach and illustrates the power of isogenic models. Furthermore, the NOTCH pathway interaction with Aurora kinase inhibitors provides an example of how 'guilt by association' can shed light on drug-action mechanism or function of cancer genes[18]. In summary, the ability to efficiently measure large numbers of drug-gene interactions in human cells has the potential to provide insight into various aspects of chemical biology.

METHODS

Cell culture, antibodies, compounds and RNAi. MCF10A cells (ATCC) were cultured in DMEM/F12 supplemented with 5% horse serum (Gibco), penicillin-streptomycin, insulin (10 μg ml^{-1}), cholera toxin (100 ng ml^{-1}), EGF (20 ng ml^{-1}) and hydrocortisone (500 ng ml^{-1}) (Sigma). All other cells were grown in DMEM supplemented with 10% FBS (Gibco) and penicillin-streptomycin. PDK1 antibody (E-3) and GFP- and p53-specific (DO-1) antibodies were purchased from Santa Cruz Biotechnology. Beta-actin– and c-Myc–specific antibodies were obtained from Sigma-Aldrich. All other antibodies were acquired from Cell Signaling Technology. Compounds were obtained from SynThesis Medchem (China) except for rapamycin, everolimus, mitomycin C and PP242 (Sigma). Compound purity was ≥95% according to the manufacturer except for PP242 (≥98%). The γ-secretase inhibitor dibenzazepine was kindly provided by J. Bradner (Dana-Farber Cancer Institute), and the purity and identity of this compound were verified by MS and matched published standards.

siRNA experiments were performed by transfecting MCF10A cells with siLentfect (Bio-Rad) and 10 nM siRNA. c-MYC siRNA SMARTPool sequences (Dharmacon) were: 5′-CGAUGUUGUUUCUGUGGAA-3′, 5′-AACGUUAGCUUCACCAACA-3′, 5′-GAACACACAACGUCUUGGA-3′, 5′-ACGGAACUCUUGUGCGUAA-3′; the sequence for Luciferase was: 5′-UCGAAGUAUUCCGCGUACG-3′. The previously validated shRNA-targeting mTOR was obtained by cloning oligos into pLKO.1 and was verified by sequencing[41].

Barcoded vectors and generation of isogenic cell lines. The stuffer fragment in the lentiviral vector pLKO.1 (ref. 42) was replaced with a short linker sequence and barcodes (Flexmap barcode tags; **Supplementary Table 5**) flanked by primer sites and was inserted 5′ of the U6 promoter. This vector (pLKO.2, **Supplementary Fig. 1**) was then used to introduce stable DNA barcodes into cells by lentiviral transduction. Cloning oligos into pLKO.2 using the AgeI and EcoRI restriction sites generated vectors expressing short hairpin RNA. An overview of all vectors used in the screen is provided in **Supplementary Table 1**.

MCF10A isogenic cell lines overexpressing cDNAs or shRNAs were produced by lenti- or retroviral transduction and selection. Stable lines were cultured for approximately 4 weeks before the screen and were barcoded by a second infection, when applicable. Prior to siRNA SMARTPool transfections MCF10A were infected with barcoded lentivirus.

Screen setup and Luminex assay. For each compound, a 4-point dose-response curve was determined in MCF10A cells using the Celltiter Glo assay (Promega). From these data, concentrations were selected for the screen. All barcoded cell lines were pooled, counted and seeded in multiwell plates in quadruplicate. Compound or DMSO was added 16 h after seeding using a liquid-handling robot (Cybio). Medium was refreshed every second day, and cells were cultured for a total of 9 d (split once), after which genomic DNA was isolated and barcodes were amplified. Genomic DNA extraction was performed with a liquid handler (Cybio) using the Genfind v2.0 kit (Agencourt). In brief, medium was removed and cells were washed twice with PBS. After lysis (1% SDS, 10 mM EDTA, 10 mM NaCl and 10 mM Tris-HCl, pH 8.0,), 100 μl raw lysate was transferred into 96-deep-well plates and 60 μl Agencourt binding buffer was added. Beads were washed six times with 70% ethanol and purified genomic DNA was eluted in distilled H_2O. Barcodes were amplified in a two-step protocol by PCR (forward: 5′-CGATTAGTGAACGGATCTC-3′; reverse: 5′-GAAGGTGAGAACAGGAGC-3′), and linear amplification was performed with a 5′ biotinylated primer (5′biotin-TGAGGATAGCAGAGAAGG-3′). The single-stranded product was hybridized to precoupled Luminex xMAP beads (as described[43]) for 1.5 h at 40 °C in 384-well plates, and streptavidin-coupled phycoerythrin (SAPE, Invitrogen) was added for 30 min at 40 °C. Finally, beads were washed once, and samples were measured in a Flexmap 3D plate reader (Luminex) at 40 °C.

Quantitative real-time PCR. RNA was isolated from subconfluent cells using Trizol (Invitrogen). After purification and DNase treatment (Turbo-DNA free, Ambion), reverse transcription was performed using random hexamer primers and RevertAid reverse transcriptase (Fermentas). Quantitative real-time PCR was carried out using the iTaq SYBR Green Supermix (Bio-Rad) according to manufacturer instructions. Measurements were performed in triplicate and related to *GAPDH* as a reference gene. All primer sequences are listed in **Supplementary Table 6**.

GFP competition assay. Cells were infected with vectors carrying the cDNAs for ICN1 and GFP (EF-hICN1-CMV-GFP) or an empty control vector. After infection, cells were pooled and distributed among multiple six-well plates for BEZ-235 or DMSO treatment. GFP-positive cells were measured by FACS or microscopy (Leica DMI6000B). For the microscopy analysis, ten randomly chosen fields were imaged for each cell line–drug combination, and cells were quantified using CellProfiler (The Broad Institute). Uninfected cells were used to determine the amount of background fluorescence.

Oncomine analysis. The Wooster cell-line dataset consists of over 300 cell lines (GSK300) that have been profiled for gene expression, copy number (CHG) and sensitivity to 19 compounds, including the PI3K-mTOR inhibitors BEZ-235, GSK1059615, temsirolimus and the Aurora kinase inhibitor GSK1070916 (additional information at https://cabig.nci.nih.gov/caArray_GSKdata/). The analysis was done by grouping the drugs based on target pathway (that is, PI3K-mTOR or Aurora kinase). A c-MYC copy number >4 was considered evidence for c-MYC gene amplification and the classification as resistant or sensitive, and median NUMB expression was used as defined by Oncomine.

Statistical analysis. Tests for statistical significance (as indicated in the text) and distribution of the data (Kolmogorov-Smirnov test) were calculated in GraphPad Prism 5.0 (GraphPad software). Experiments were performed in triplicate unless otherwise noted. $P < 0.05$ ($\alpha = 5\%$) was accepted as statistically significant.

Further information on screen data analysis can be found in **Supplementary Methods**.

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Author Contributions

S.M.B.N. and M.K.M. conceived the study, designed experiments, analyzed data and wrote the manuscript. M.K.M. and S.M.B.N., with help from I.Z.U. and N.C.-M., set up the multiplexing assay. B.V.G., I.Z.U and M.K.M. created and characterized the isogenic cell lines. J.C. and G.D. designed the analysis platform and database infrastructure for the screen. G.D. and M.K.M. analyzed the screening data and wrote R code to identify hits. M.K.M. performed the majority of experiments. C.K., M.S., H.L. and S.M.B.N. performed and helped with additional experiments.

Competing financial interests

The authors declare no competing financial interests.

Additional information

Supplementary information is available online at http://www.nature.com/naturechemicalbiology/. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html. Correspondence and requests for materials should be addressed to S.M.B.N.

SUPPLEMENTARY INFORMATION

A chemical genetic screen reveals a resistance mechanism to PI3K inhibitors in cancer

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Supplementary Methods

Luminex data analysis

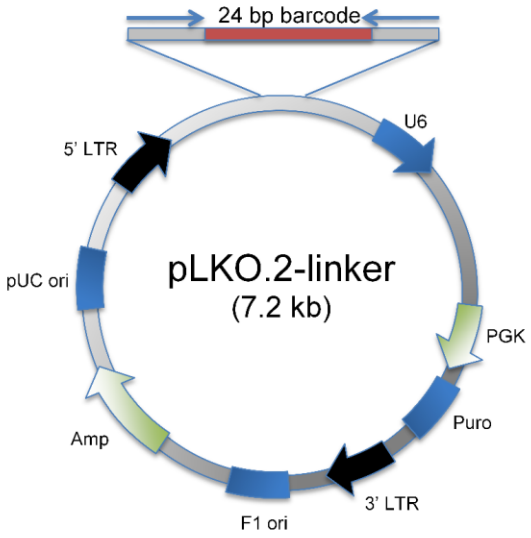
Raw bead signal intensities were obtained for each barcode, i.e. gene, and well, i.e. drug treatment. The multiple measurements performed by the instrument for each barcode in each well were replaced by their medians.

The barcode median values from each well were then normalized by a factor such that their sum was identical in all the wells. All the normalized medians were then log-transformed. A first robust linear regression was computed according to the additive model data ~well+barcode+residual (Wilkinson-Rogers notation), where "data" represents the log-transformed normalized medians, "well" is the well-specific deviation, "barcode" is the barcode-specific deviation, and "residual" is the residual data variability. This regression provided the final data normalization step by independently removing the well and barcode systematic biases, and its residuals were submitted to a second robust linear regression to deconvolute the well-barcode interactions. This second regression model naturally includes all the possible combinations of barcode and well effects (factor crossing) to explain the remaining residuals residual ~well×barcode+error. The P-values of this second regression coefficients were used to rank the interactions, whereas the regression coefficients themselves estimated the magnitude of the well-barcode interactions. A detailed outline of the algorithm will be published elsewhere (Duernberger *et al.*, in preparation).

Resistance scores calculation

Resistance scores were calculated by first excluding all normalized data points in the sensitivity direction. Next, the fold change (drug vs. all) was calculated for each drug treatment of ICN1, c-MYC and MDM2 as a control gene. The mean resistance scores were then calculated by taking all PI3K inhibitor data (minus BEZ-235) or all remaining drugs. A table with the top 25 drug-gene hits in the screen is availble (Supplementary Table 1).

Supplementary Results



pLKO.2-linker vector was generated as follows: The 5' NotI site in pLKO.1-stuffer [Cell. 2006 Mar 24;124(6):1283-98] was destroyed and oligos containing the barcode cloning site was ligated into the now unique NotI site 5' from the U6 promoter.

Inserted verified sequence:
GGCCCGGGGCTAGCTGAGGATAGCAGAGAAGGACGCGTGATCCTGCAGCTCC
TGTTCTCACCTTCGCGGCCGCTCTAGAGTCGACTACGTACGGGGC

A second sequence containing various RE sites was cloned 3' from the stuffer into the EcoRI site, leaving the 5' site intact.

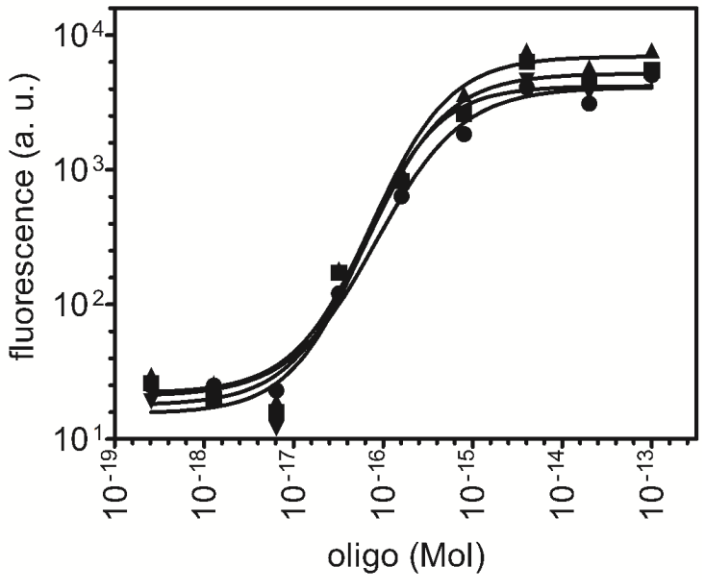
Inserted verified sequence:
AATTCTACGTAGATATCTGATCACCATACGTATGGCCCGGGT

Finally, the stuffer sequence was removed and a short linker sequence containing a RNA polymerase III termination sequence was inserted: TGATCTTTTTGATCGATC

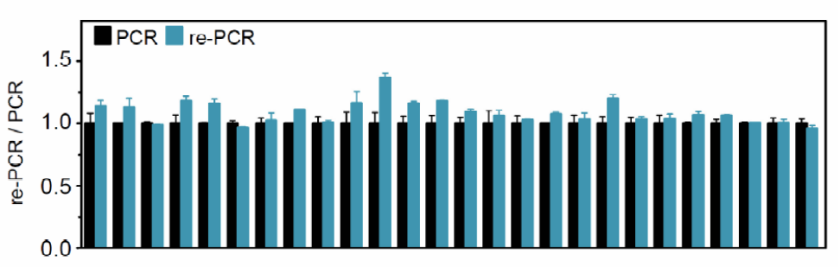
Barcode sequences were cloned into MluI/PstI sites. Hairpin oligos can be cloned into AgeI/EcoRI sites.

Supplementary Figure 1 | The pLKO.2 vector map. Cloning strategy, vector map

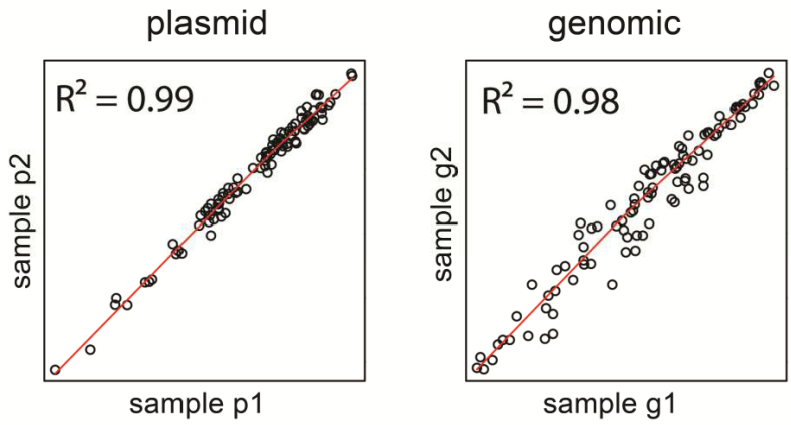
and sequence.



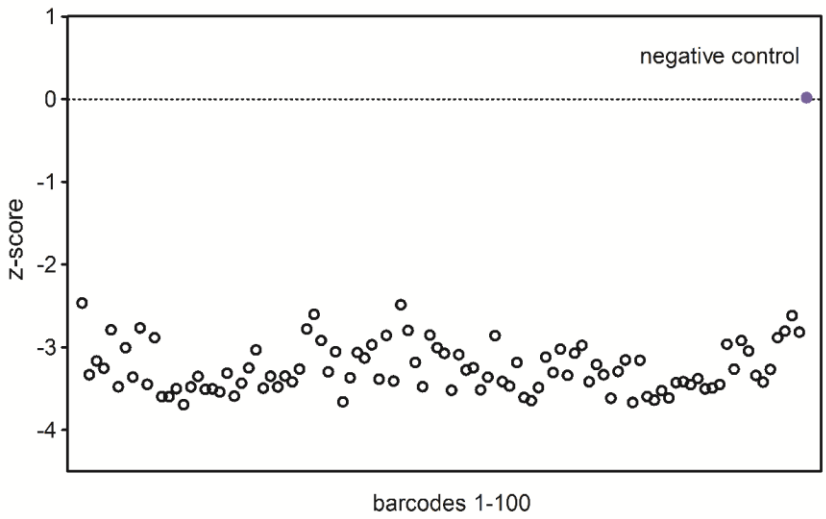
Supplementary Figure 2 | Luminex signal response curve. 5' biotinylated DNA oligos representing the linear amplification (LAMP) product were hybridized to xMAP microspheres at the indicated concentrations and fluorescent intensities were measured. Shown are the results for four independent barcode sequences.



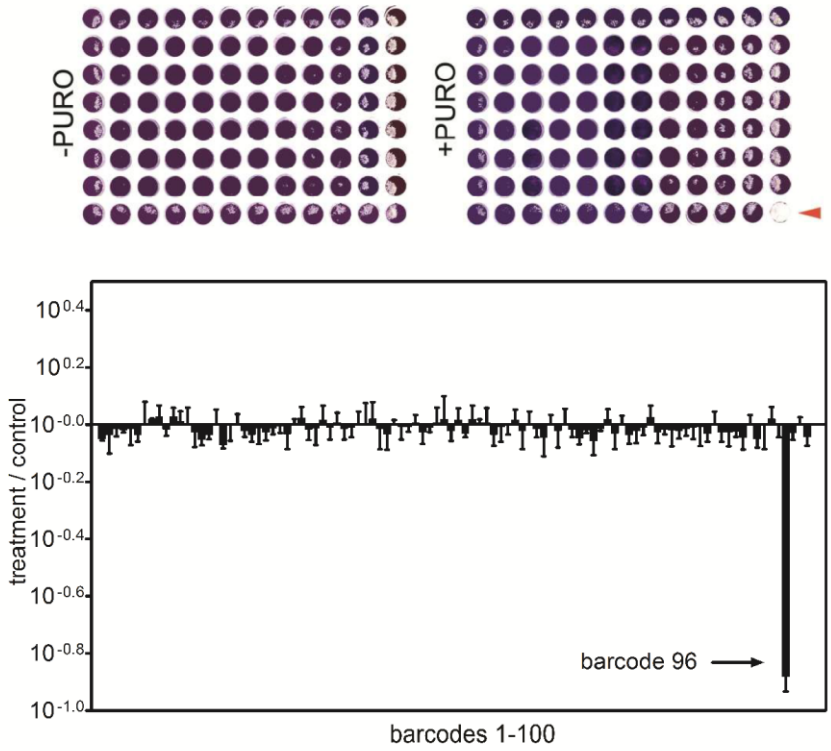
Supplementary Figure 3 | PCR of barcodes shows limited PCR bias. PCR product of a pool of barcoded vectors (PCR, black bars) was used for a nested second PCR (re-PCR, blue bars) and fluorescent intensities were measured. Signals were normalized to the first PCR and the experiment was performed in triplicate. Error bars represent standard deviations.



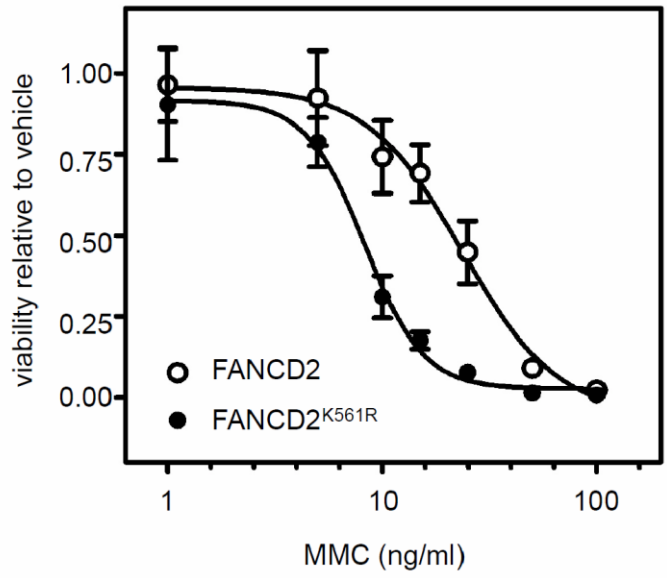
Supplementary Figure 4 | Sample to sample variation. Scatter plots of barcode signals derived from a pool of barcoded pLKO.2 vectors (left) or genomic DNA derived from HeLa cells infected with the same pool (right). Pearson correlation coefficients were calculated and shown are representative examples of three independent experiments.



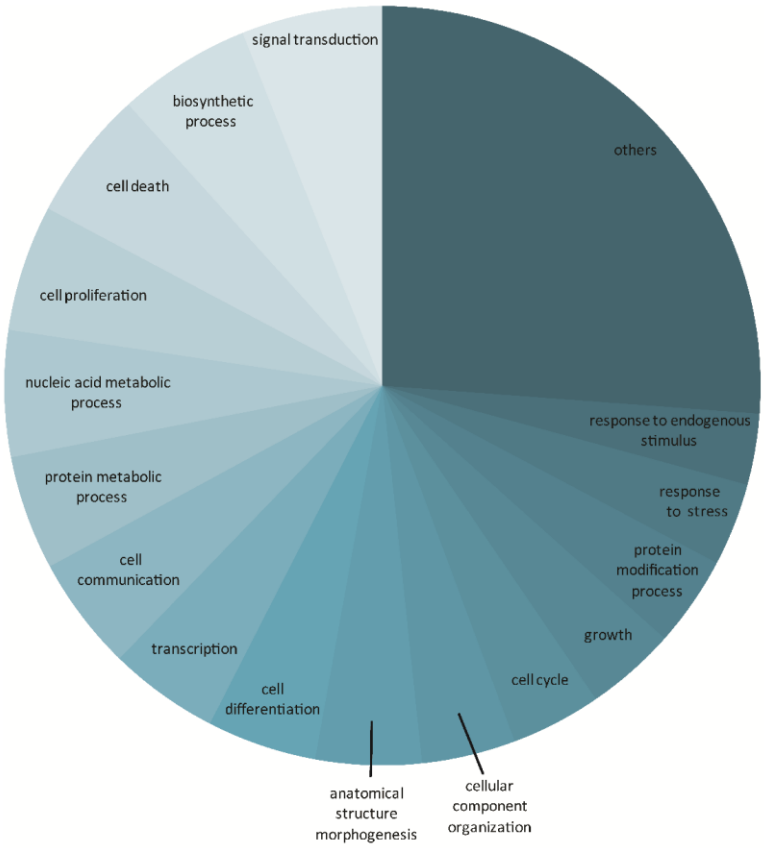
Supplementary Figure 5 | The assay shows low cross-hybridization between barcodes. One hundred pools with 99 barcoded plasmids (each missing a different barcode) were quantified using the Luminex assay. For each measurement the z-score (standard score) was calculated for the missing barcode by subtracting the mean and dividing by the standard deviation.



Supplementary Figure 6 | Dropout experiment with barcoded cells. MCF10A cells were infected with 96 distinct barcoded lentiviral vectors one of which lacking the puromycin resistance gene (indicated with arrow) and treated with puromycin or left untreated (upper panels). Barcodes amplified from pooled cells subjected to puromycin selection for 3 days were analyzed and compared to unselected cells (lower panel). The experiment was performed in quadruplicate and standard deviations are indicated.

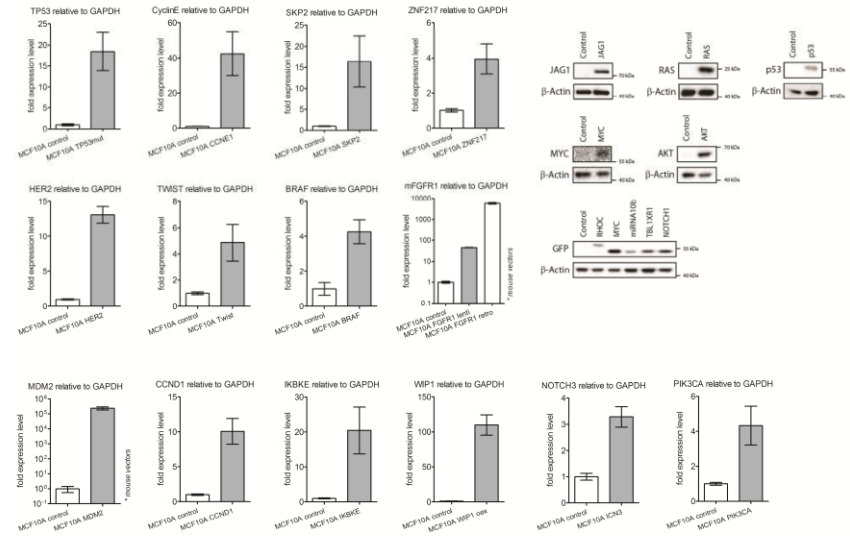


Supplementary Figure 7 | FANCD2-K561R cells display increased sensitivity to Mitomycin C. Indicated cells were treated with MMC or left untreated for 5 days. Relative cell viability was measured using Cell titer Glo and shown are the means and standard deviations of a triplicate experiment.

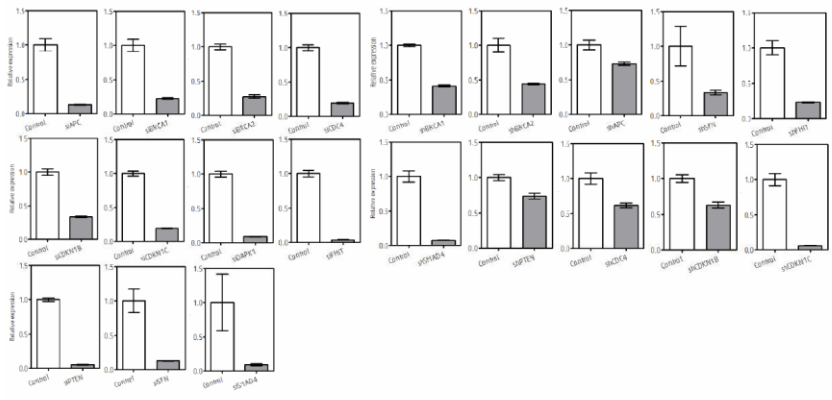


Supplementary Figure 8 | Breast cancer gene set. Gene ontology annotation of the seventy breast cancer genes selected for the screen.

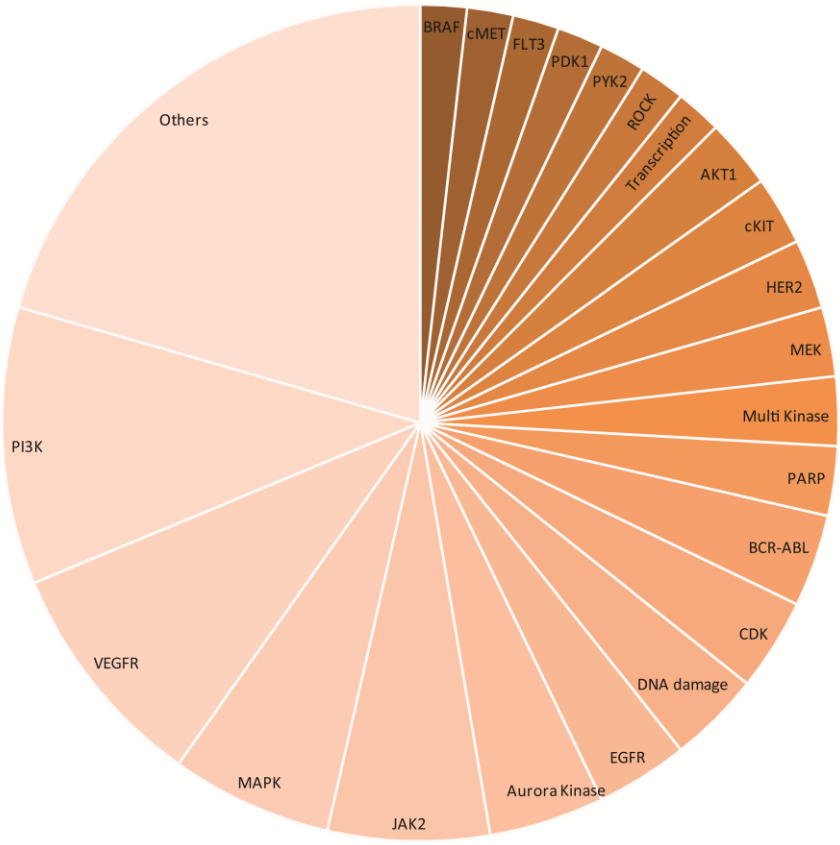
a



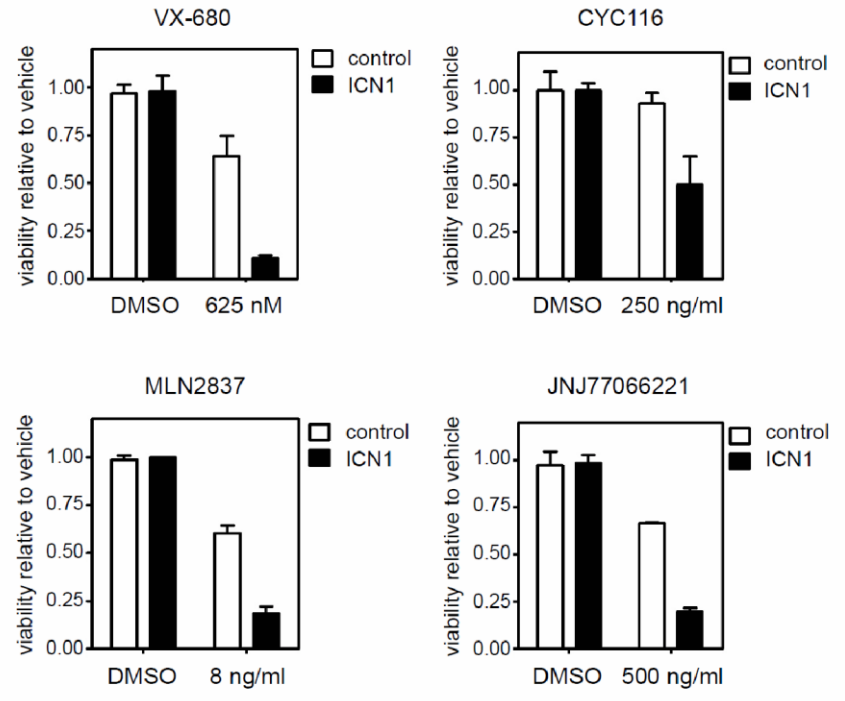
b



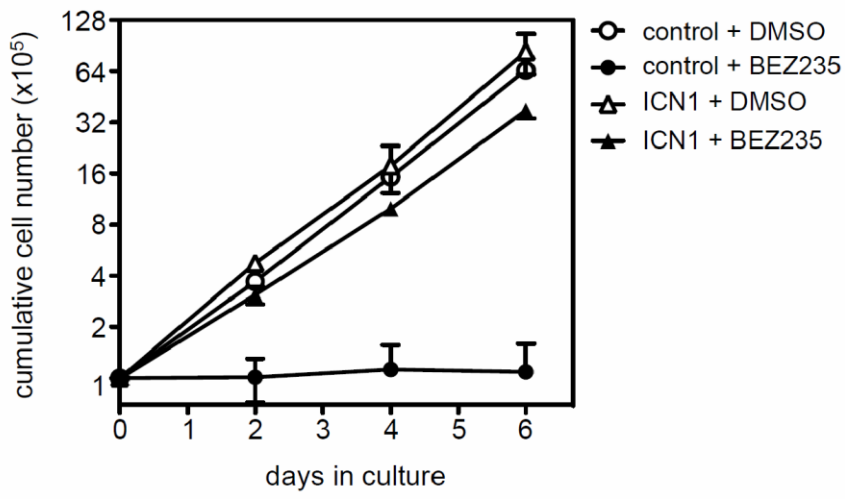
Supplementary Figure 9 | Validation of cDNA and shRNA expressing MCF10A cells. (a, b) Protein or mRNA from cDNA **(a)** or shRNA **(b)** infected and selected cells were analyzed by Western blot or qRT-PCR as indicated.



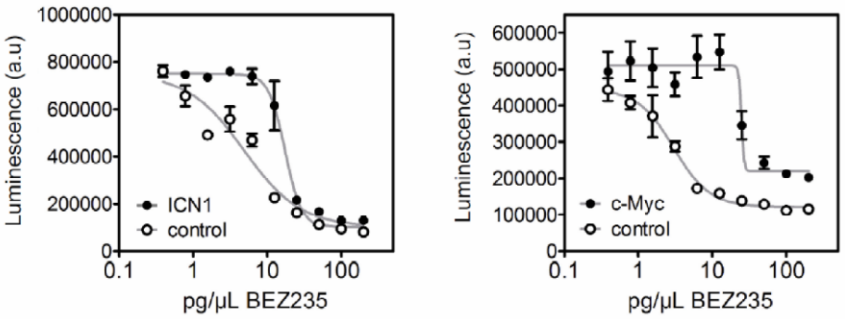
Supplementary Figure 10 | Drug targets. Overview of the primary targets of the drug library used in the screen.



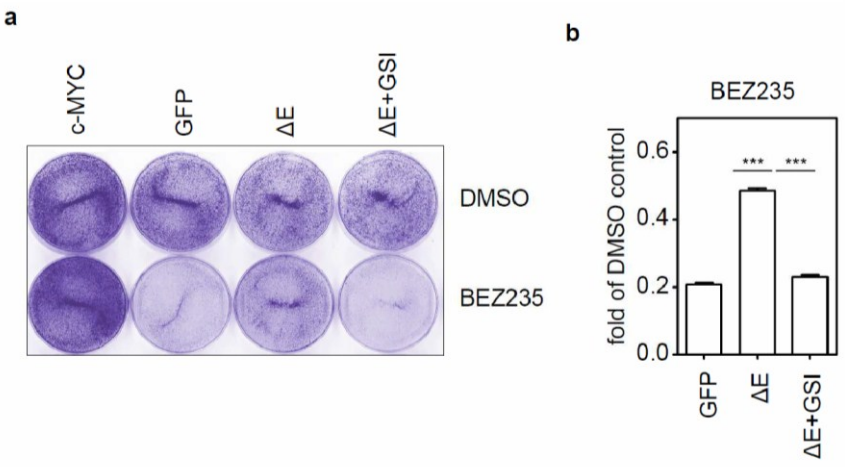
Supplementary Figure 11 | ICN1 expressing MCF10A cells display synthetic lethal interaction with Aurora kinase inhibitors. Cells were treated with the indicated compounds for 4 days and cell viability was measured using Cell Titer Glo. The experiments were performed in triplicate (standard deviation is indicated) and shown is a representative example of at least two independent experiments.



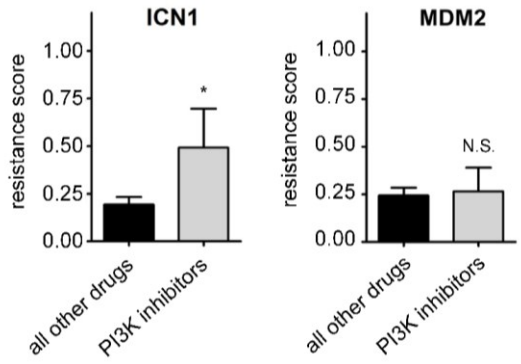
Supplementary Figure 12 | ICN1 confers resistance to BEZ-235. Indicated cells were grown in the presence or absence of BEZ-235 (20pg/ul) and viable cell numbers were determined.



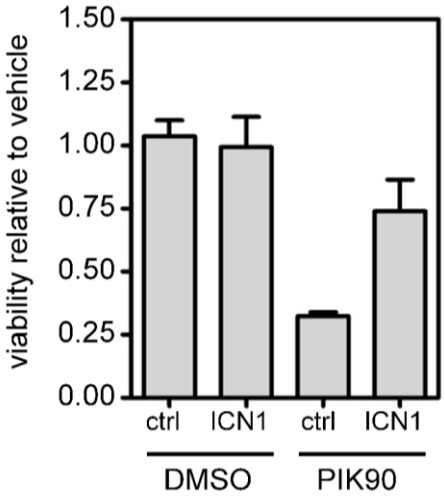
Supplementary Figure 13 | MCF10A cells expressing ICN1 or c-MYC are resistant to BEZ-235. Raw CellTiter Glo data of two representative dose-respone experiments.



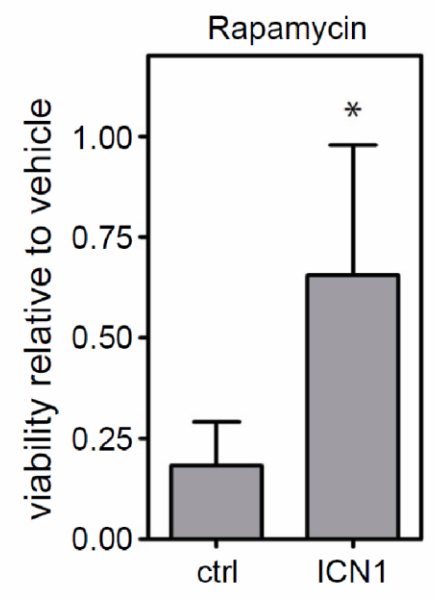
Supplementary Figure 14 | A constitutive active NOTCH1 mutant lacking the extracellular domain (delta E) confers resistance to BEZ-235.(a) MCF10A cells were transfected with the indicated plasmids and treated with DMSO, BEZ-235 (20 pg/ul) or BEZ-235 and the gamma secretase inhibitor dibenzazepine (1uM). After transfection, cells were seeded at low density and stained with crystal violet after 7 days. Gamma secretase inhibition alone did not affect cell growth (not shown). **(b)** MCF10A cells were transfected and treated as indicated for 4 days. Crystal violet stained dishes were quantified after 7 days (*** indicates p<0.001).



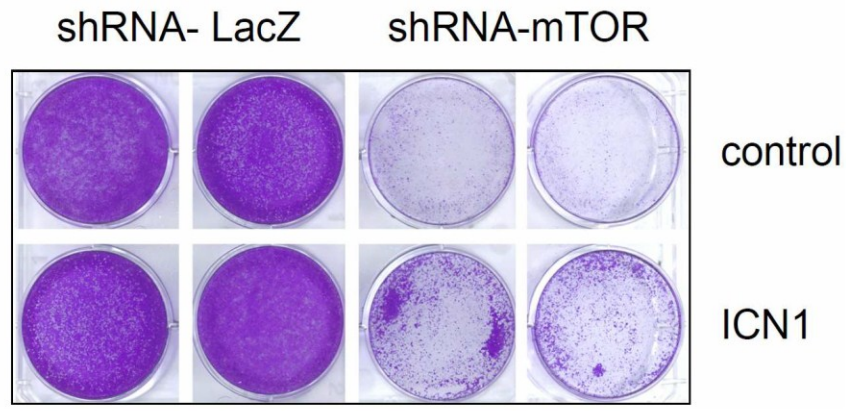
Supplementary Figure 15 | Combined PI3K inhibitors also show resistance with ICN1. The mean resistance scores (see Methods) for all ICN1 drug treatments were divided into PI3K and non-PI3K inhibitors and the mean for each group was calculated (* p=0.02, one-sided Mann-Whitney test). MDM2 drug treatments served as a control. BEZ-235 was left out of the analysis.



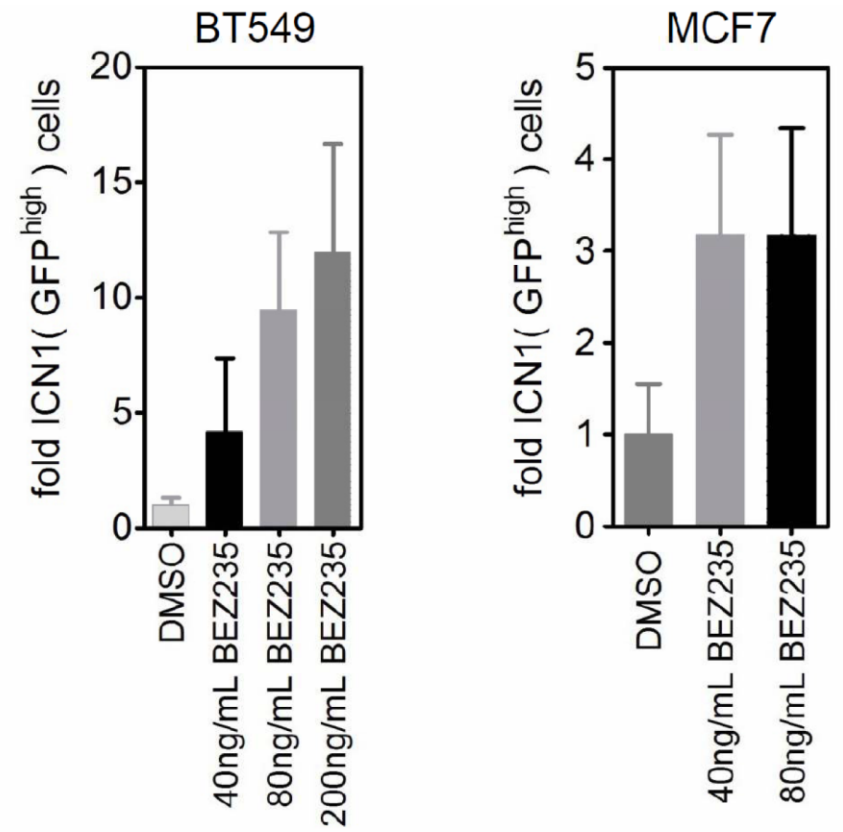
Supplementary Figure 16 | ICN1 also confers resistance to PIK90. Bar graph showing relative viability of ICN1 or control MCF10A cells treated with PIK90 (250ng/ml) or vehicle for 5 days. Standard deviations of three replicates are indicated.



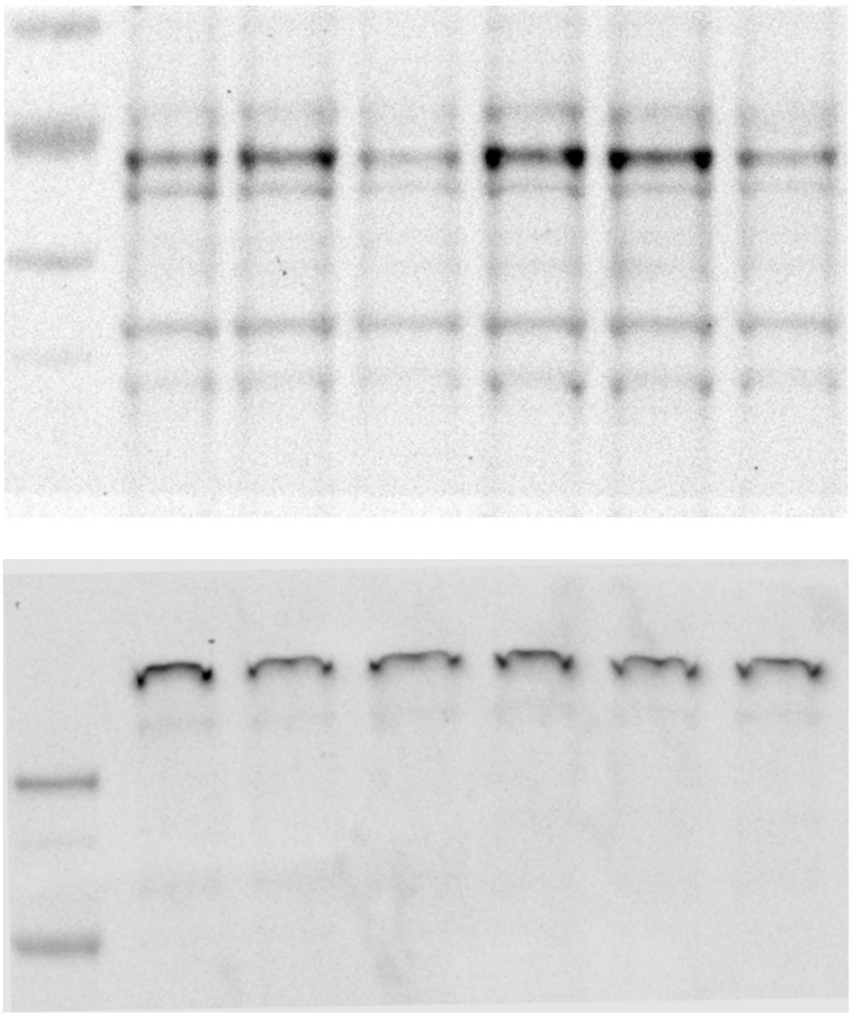
Supplementary Figure 17 | MCF10A cells expressing ICN1 are resistant to rapamycin. Indicated MCF10A cells were treated for 5 days with 10 uM rapamycin. Shown is the mean of a representative experiment performed in triplicate (* $p<0.05$).



Supplementary Figure 18 | ICN1 expressing cells are less sensitive to inhibiton of mTOR by RNAi. Control or ICN1 expressing MCF10A cells were infected in duplicate with lentivirus expressing an shRNA against LacZ or mTOR. Cells were selected and stained with crystal violet after 7 days.

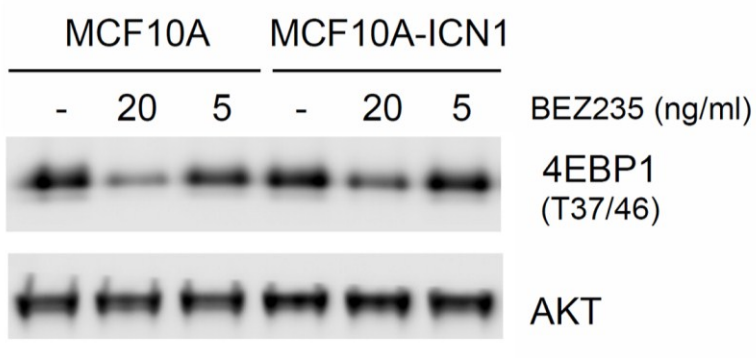


Supplementary Figure 19 | ICN1 confers resistance to BEZ-235 in the breast cancer cell lines BT549 and MCF7. BT549 or MCF7 cells were infected with a lentivirus expressing ICN1 and GFP and treated as indicated for 3 days after which the fraction of GFP positive cells was determined (also see Methods). Plotted is the fold increase in GFP positive cells (GFP-high) compared to day 0 and standard devations of three independent measurements.

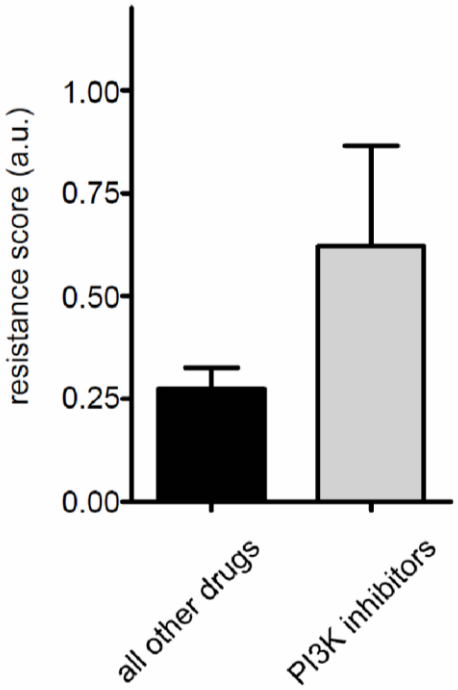


Supplementary Figure 20 | BEZ235 lowers pS6K T371 irrespective of ICN1.

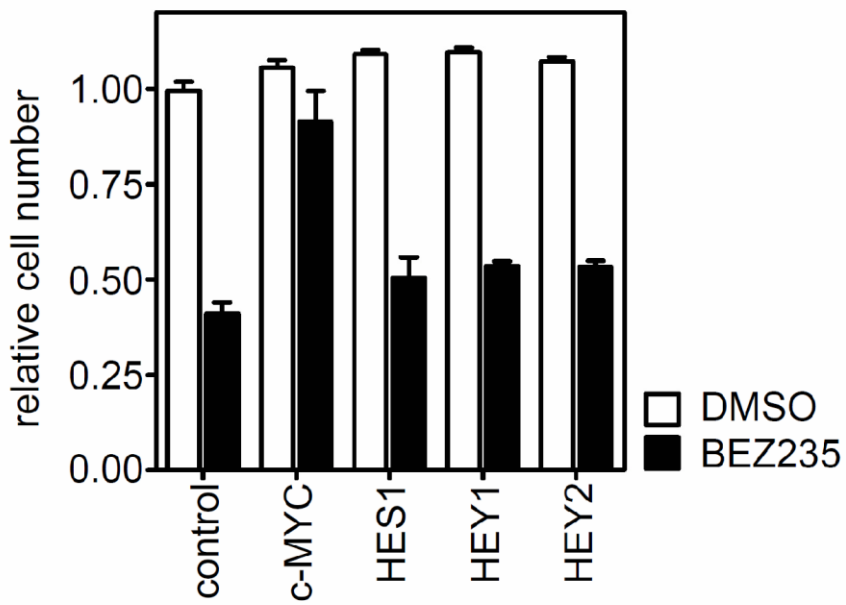
Uncropped version of the pS6K T371 (upper panel) and mTOR (lower panel; loading control) western blots from 4a.



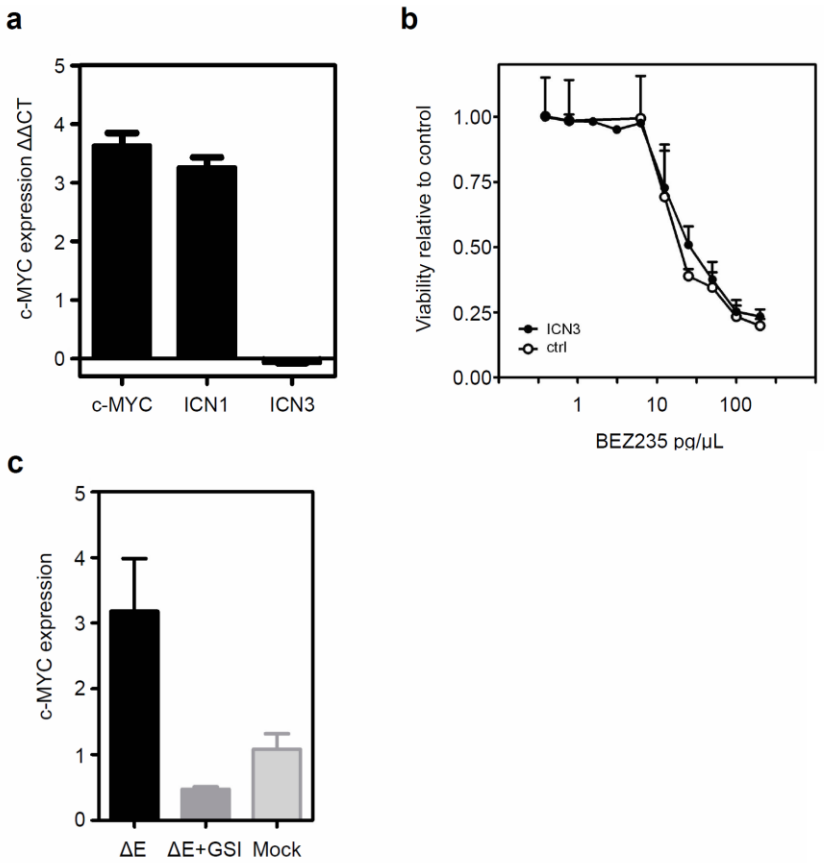
Supplementary Figure 21 | Phosphorylation of the mTORC1 downstream target 4EBP1 is inhibited in MCF10A cells expressing ICN1. Western blot analysis of cells treated with BEZ-235 for 6 hours. AKT served as a loading control.



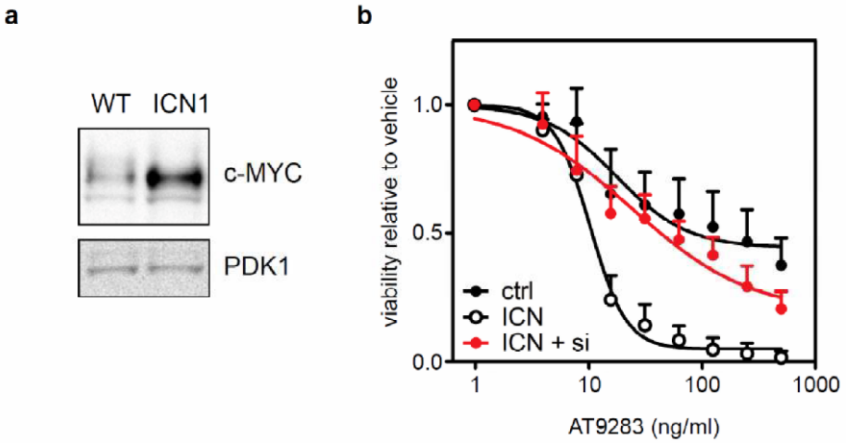
Supplementary Figure 22 | MCF10A-MYC cells show trend towards resistance to PI3K inhibitors in the screen. The mean resistance scores (see Methods) for all c-MYC drug treatments were divided into PI3K and non-PI3K inhibitors and the mean for each group was calculated. BEZ-235 was left out of the analysis (P= 0.08).



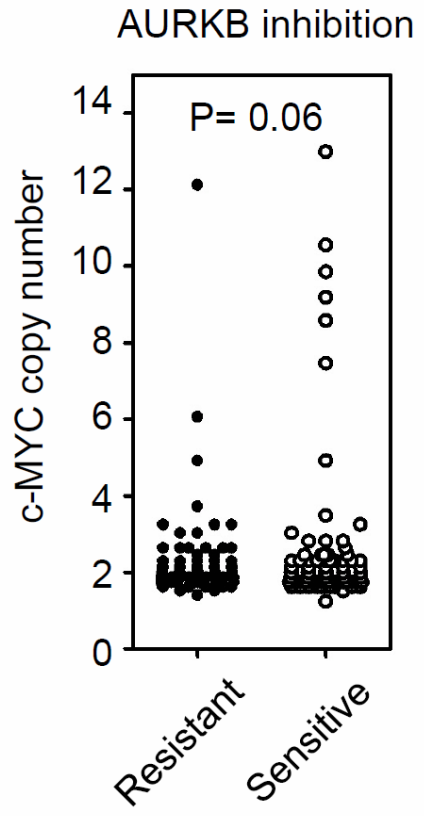
Supplementary Figure 23 | NOTCH target genes HES1, HEY1 and HEY2 do not confer resistance to BEZ-235. MCF10A cells were transfected with the indicated plasmids and treated with BEZ-235 for 5 days. Crystal violet stained dishes were quantified and shown is the average of a triplicate experiment with standard deviations.



Supplementary Figure 24 | c-Myc expression correlates with resistance to BEZ-235 in cells expressing ICN3 or NOTCH1 deltaE mutant. a) qRT-PCR for c-MYC expression levels in MCF10A cells expressing c-MYC, ICN1 or ICN3. Values are relative to control MCF10A cells. Error bars indicate standard devations. **b)** Dose response curve. Cells were treated with BEZ-235 and cell viability was measured 5 days later. Standard devations are indicated. **c)** Quantitative RT-PCR based c-MYC expression levels in MCF10A cells expressing deltaE NOTCH1 treated with 1.0 uM dibenzazepine or DMSO as a control. Mock indicates wild type MCF10A cells exposed to transfection agent with no exogenous DNA added.



Supplementary Figure 25 | c-Myc protein is upregulated in ICN1 cells and knockdown rescues synthetic lethality with Aurora kinase inhibition. a) Western blot for c-MYC protein in control MCF10A or MCF10A-ICN1 cells. PDK1 served as a loading control. **b)** Dose-response curve with AT9283 of MCF10A control or ICN1 cells transfected with 5nM Luciferase siRNA (ctrl, ICN1) or ICN1 cells transfected with 5nM c-MYC siRNA (ICN1+si). The graph is the combined result of four independent experiments performed in triplicate and standard deviations are indicated.



Supplementary Figure 26 | Oncomine c-MYC copy number analysis for cells lines treated with the Aurora kinase inhibitor GSK1070916. Cell lines are divided into resistant (IC50 > 318 nM) or sensitive (IC50< 99 nM), as defined by Oncomine. Chi square P value is indicated.

Number	Name	Type	Aberrant (%)	References
1	AKT1	mutated	6.8	http://www.ncbi.nlm.nih.gov/pubmed/19706770
2	ANXA1	downreg	50	http://www.ncbi.nlm.nih.gov/pubmed/18776816
3	APC	mutated	4.03	ROCK-BCFG
4	B-RAF	mutated	4.91	ROCK-BCFG
5	BRCA1	mutated	1	NA
6	BRCA2	mutated	1	NA
7	c-MYC	amplified	15.7	http://www.ncbi.nlm.nih.gov/pubmed/11104567
8	CADM1	methylated	48	http://matrix.ugent.be/temp/static/
9	CCND1	amplified	20	http://www.ncbi.nlm.nih.gov/pubmed/19636701
10	CCNE1	amplified	6	http://www.ncbi.nlm.nih.gov/pubmed/15682439
11	CDC4[a]	mutated	NA	http://www.ncbi.nlm.nih.gov/pubmed/14871801?dopt=Abstract
12	CDH1	mutated	21.28	ROCK-BCFG
13	CDH13	methylated	33	http://matrix.ugent.be/temp/static/
14	CDKN1B	downreg	13	http://annonc.oxfordjournals.org/cgi/content/abstract/19/4/660
15	CDKN1C	downreg	50	http://www.biomedcentral.com/1471-2407/8/68
16	CDKN2A[b]	mutated	5.28	ROCK-BCFG
17	CTNNB1	mutated	4.73	ROCK-BCFG
18	DAPK1	methylated	10	http://matrix.ugent.be/temp/static/
19	DBC1[c]	deleted	1	http://www.pnas.org/content/99/21/13647.full
20	DBC2[d]	deleted	3.5	http://www.pnas.org/content/99/21/13647.full
21	DEAR1	mutated	13	http://www.plosmedicine.org/article/info:doi%2F10.1371%2Fjournal.pmed.1000068
22	EGR3[e]	deleted	2.5	http://www.pnas.org/content/99/21/13647.full
23	ERBB2/HER2	amplified	20	http://www.ncbi.nlm.nih.gov/pubmed/7908410
24	ESR1	methylated	25	http://matrix.ugent.be/temp/static/
25	ESR2	methylated	25	http://matrix.ugent.be/temp/static/
26	FGFR1	amplified	8.7	http://www.ncbi.nlm.nih.gov/pubmed/17397528
27	FHIT	methylated	10	http://matrix.ugent.be/temp/static/
28	GSTP1	methylated	6	http://matrix.ugent.be/temp/static/
29	HDM2	amplified	7	http://www.ncbi.nlm.nih.gov:80/pmc/articles/PMC2033791/
30	HIC-1	methylated	50	http://matrix.ugent.be/temp/static/
31	IKBKE	amplified	16.3	http://www.ncbi.nlm.nih.gov/pubmed/17574021
32	JAG1	upreg	NA	http://www.ncbi.nlm.nih.gov/pubmed/16166334
33	K-RAS	mutated	5.56	ROCK-BCFG
34	KLF17	downreg	NA	http://www.ncbi.nlm.nih.gov/pubmed/19801974
35	MAP2K4	mutated	3.73	ROCK-BCFG
36	MINPP1[f]	deleted	ND	NA
37	miR-10b	upreg	NA	http://www.ncbi.nlm.nih.gov/pubmed/17898713
38	MTAP[g]	deleted	ND	http://cancerres.aacrjournals.org/cgi/reprint/62/22/6639
39	NOTCH1	active	NA	http://www.ncbi.nlm.nih.gov/pubmed/16166334
40	NOTCH3	amplified	1	http://cancerres.aacrjournals.org/cgi/content/abstract/68/6/1881
41	PALB2	mutated	1	NA
42	PCTH1	mutated	1	ROCK-BCFG
43	PIK3CA	mutated	24.71	ROCK-BCFG
44	PPP2R1A[h]	mutated	5	http://www.ncbi.nlm.nih.gov/pubmed/10713707
45	PPP2R1B[i]	mutated	5	http://www.ncbi.nlm.nih.gov/pubmed/10713707
46	PTEN[j]	mutated	5.45	ROCK-BCFG
47	PTGS2	methylated	25	http://matrix.ugent.be/temp/static/
48	PYCARD	methylated	10	http://matrix.ugent.be/temp/static/
49	RARb	methylated	10	http://matrix.ugent.be/temp/static/
50	RASSF1A	methylated	10	http://matrix.ugent.be/temp/static/
51	RB1	mutated	13.85	ROCK-BCFG
52	RBSP3	methylated	NA	http://www3.interscience.wiley.com/journal/121452012/abstract?CRETRY=1&SRETRY=0
53	RHOC	upreg	NA	http://www.ncbi.nlm.nih.gov/pubmed/12237774
54	RUNX3	methylated	25	http://matrix.ugent.be/temp/static/
55	SCGB3A1	methylated	57	http://matrix.ugent.be/temp/static/
56	SFN	methylated	50	http://www.ncbi.nlm.nih.gov/pubmed/11423985
57	SFRP1	methylated	15	http://matrix.ugent.be/temp/static/
58	SKP2	upreg	13	http://annonc.oxfordjournals.org/cgi/content/abstract/19/4/660
59	SLIT2	methylated	58	http://matrix.ugent.be/temp/static/
60	SMAD4[k]	mutated	4.62	ROCK-BCFG
61	SOCS1	methylated	23	http://www.ncbi.nlm.nih.gov/pubmed/15361843
62	SYK	methylated	25	http://matrix.ugent.be/temp/static/
63	TBL1XR1	amplified	1.8	http://www.ncbi.nlm.nih.gov/pubmed/19706770
64	TIMP3	methylated	20	http://matrix.ugent.be/temp/static/
65	TNFRSF10A	deleted	3.5	http://www.pnas.org/content/99/21/13647.full

66	TP53	mutated	59.02	ROCK-BCFG
67	TWIST	upreg	NA	http://www.ncbi.nlm.nih.gov/pubmed/15864483
68	WIF1	methylated	60	http://matrix.ugent.be/temp/static/
69	WIP1	upreg	NA	http://www.ncbi.nlm.nih.gov/pubmed/16897432
70	ZNF217	amplified	NA	http://cancerres.aacrjournals.org/cgi/content/full/61/4/1250

Oncogene
Tumor supressor
Passenger

(NA= not available)

Comment

a from cell line data
also found homozygously
b deleted
c passenger
d also found mutated
e passenger
f passenger with PTEN deletions
g passenger with INK4A deletions
h no functional validation
i no functional validation
also found homozygously
j deleted
also found homozygously
k deleted

Supplementary Table 1 | Genes selected for the breast cancer screen

Name	Type	Used vector	Barcodes
FGFR1	ampl	pBABEpuro-Fgfr1	16
AKT1	mut	pMIG-Myr-AKT	30
B-RAF	mut	pBABEpuro-BRAF-600F	5,6
CCND1	ampl	pBABEhygro-CyclinD1	21
CCNE1	ampl	pBABEpuro-CyclinE	4
ERBB2/HER2	ampl	pQCXIP-HER2	48
HDM2	ampl	pBABEpuro-Mdm2	24
IKBKE	ampl	pBabe-Neo-Flag-IKBKE	19
JAG1	upreg	pBABEpuro-Jag1-HA	32
H-RAS	mut	pBABEpuro-H-RAS-V12G	31
miR-10b	upreg	MDH1-PGK-GFP miRNA10b	22
c-MYC	ampl	pMIG-c-Myc	23
NOTCH1	active	EF-hICN1-CMV-GFP	44
NOTCH3	ampl	pBABEpuro-Icn3-HA	39
PIK3CA	mut	pBABEpuro HA PIK3CA H1047R	14
RHOC	upreg	pMIG-RhoC	29
SKP2	upreg	pBABEpuro-myc-SKP2	9
TBL1XR1	ampl	pMIG-TLR1	40
TP53	mut	pBABEpuro-p53 R248W	7,12
TWIST	upreg	pBABEpuro-mTwist	1
WIP1	upreg	pBABEpuro-Wip1	3
ZNF217	ampl	pLNCX-ZNF217-HA	25

Supplementary Table 2 | Selected breast cancer genes for overexpression

Number	Name	Type	Dharmacon OTP pool
23	ANXA1	downreg	yes
24	APC	mutated	yes
25	BRCA1	mutated	yes
26	BRCA2	mutated	yes
27	CADM1	methylated	yes
28	CDC4	mutated	yes
29	CDH1	mutated	yes
30	CDH13	methylated	yes
31	CDKN1B	downreg	yes
32	CDKN1C	downreg	yes
33	CDKN2A	mutated	yes
34	CTNNB1	mutated	yes
35	DAPK1	methylated	yes
36	DBC1	deleted	yes
37	DBC2	deleted	yes
38	DEAR1	mutated	yes
39	EGR3	deleted	yes
40	ESR1	methylated	yes
41	ESR2	methylated	yes
42	FHIT	methylated	yes
43	GSTP1	methylated	yes
44	HIC-1	methylated	yes
45	KLF17	downreg	yes
46	MAP2K4	mutated	yes
47	MINPP1	deleted	yes
48	MTAP	deleted	yes
49	PALB2	mutated	yes
50	PCTH1	mutated	yes
51	PPP2R1A	mutated	yes
52	PPP2R1B	mutated	yes
53	PTEN	mutated	yes
54	PTGS2	methylated	yes
55	PYCARD	methylated	yes
56	RARb	methylated	yes
57	RASSF1A	methylated	yes
58	RB1	mutated	yes
59	RBSP3	methylated	yes
60	RUNX3	methylated	yes
61	SCGB3A1	methylated	yes
62	SFN	methylated	yes
63	SFRP1	methylated	yes
64	SLIT2	methylated	yes
65	SMAD4	mutated	yes
66	SOCS1	methylated	yes
67	SYK	methylated	yes
68	TIMP3	methylated	yes
69	TNFRSF10A	deleted	yes
70	WIF1	methylated	yes

Supplementary Table 3 | Selected breast cancer genes for siRNA smartpool knockdown

Name	Reference	Barcode #	Hairpin sequence
APC	TRCN0000010297	10	CCGGTAATGAACACTACAGATAGAACTCGAGTTCTATCTGTAGTGTTCATTATTTTT
BRCA1	TRCN0000039837	17	CCGGGCCTACAAGAAAGTACGAGATCTCGAGATCTCGTACTTTCTTGTAGGCTTTTT
BRCA2	Mol Cell Biol. 2004 Sep;24(17):7444-55	2	CCGGGCTCCACCCTATAATTCTGTTCAAGAGACAGAATTATAGGGTGGAGCTTTTT
BRCA2	Mol Cell Biol. 2005 Mar;25(5):1949-57	8	CCGGGGGAAACACTCAGATTAAAGATTCAAGAGATCTTTAATCTGAGTGTTTCCCTTTTTT
CDKN1B	Cancer Res. 2006 Dec 15;66(24):11623-31	47	CCGGGTACGAGTGGCAAGAGGTGTTCAAGAGACACCTCTTGCCACTCGTACTTTTTT
CDKN1C	TRCN0000039679	41	CCGGGCCTCTGATCTCCGATTTCTTCTCGAGAAGAAATCGGAGATCAGAGGCTTTTT
CDKN2A	Cancer Cell. 2003 Oct;4(4):311-9	38	CCGGCATGGTGCGCAGGTTCTTGTTCAAGAGACAAGAACCTGCGCACCATGTTTTTT
FBXW7	Science 2008 Sep; 321(5895):1499-1502	20	CCGGCAACAACGACGCCGAATTACTCGAGTAATTCGGCGTCGTTGTTGTTTTTT
FBXW7	Nat Cell Biol. 2007 Jul;9(7):765-74.	43	CCGGGTGTGGAATGCAGAGACTGGAGACTCGAGTCTCCAGTCTCTGCATTCCACACTTTTT
FBXW7	Nat Cell Biol. 2007 Jul;9(7):765-74.	46	CCGGACAGGACAGTGTTTACAAACTCGAGTTTGTAAACACTGTCCTGTTTTTTT
FHIT	TRCN0000051177	26	CCGGCCCTCTGTAGTGTTTCTCAAACTCGAGTTTGAGAAACACTACAGAGGGTTTTT
FHIT	TRCN0000051173	37	CCGGGCTGGAGACTTTCACAGGAATCTCGAGATTCCTGTGAAAGTCTCCAGCTTTTT
PTCH1	TRCN0000010497	45	CCGGTAATCCTCAACTCATGATACACTCGAGTGTATCATGAGTTGAGGATTATTTTT
PTEN	Molecular Cancer 2007, 6:16	28	CCGGAAGGCACAAGAGGCCCTAGATTTCTCGAGAAATCTAGGGCCTCTTGTGCCTTTTTTT
RB1	TRCN0000040163	11	CCGGCCACATTATTTCTAGTCCAAACTCGAGTTTGGACTAGAAATAATGTGGTTTTT
RB1	TRCN0000010418	33	CCGGACTTCTACTCGAACACGAATCTCGAGATTCGTGTTCGAGTAGAAGTTTTTT
SFN	Nature. 2007 Mar 15;446(7133):329-32	13	CCGGGGATCCCACTCTTCTTGCACTCGAGTGCAAGAAGAGTGGGATCCTTTTTT
SFN	Nature. 2007 Mar 15;446(7133):329-32	27	CCGGGTGACCATGTTTCCTCTCACTCGAGTGAGAGGAAACATGGTCACTTTTTT
SMAD4	TRCN0000040028	15	CCGGGCAGACAGAAACTGGATTAAACTCGAGTTTAATCCAGTTTCTGTCTGCTTTTT

Supplementary Table 4 | Selected breast cancer genes for shRNA knockdown

Barcode #	Sequence (sense)
1	CTTTAATCTCAATCAATACAAATC
2	CTTTATCAATACATACTACAATCA
3	TACACTTTATCAAATCTTACAATC
4	TACATTACCAATAATCTTCAAATC
5	CAATTCAAATCACAATAATCAATC
6	TCAACAATCTTTTACAATCAAATC
7	CAATTCATTTACCAATTTACCAAT
8	AATCCTTTTACATTCATTACTTAC
9	TAATCTTCTATATCAACATCTTAC
10	ATCATACATACATACAAATCTACA
11	TACAAATCATCAATCACTTTAATC
12	TACACTTTCTTTCTTTCTTTCTTT
13	CAATAAACTATACTTCTTCACTAA
14	CTACTATACATCTTACTATACTTT
15	ATACTTCATTCATTCATCAATTCA
16	AATCAATCTTCATTCAAATCATCA
17	CTTTAATCCTTTATCACTTTATCA
18	TCAAAATCTCAAATACTCAAATCA
19	TCAATCAATTACTTACTCAAATAC
20	CTTTTACAATACTTCAATACAATC
21	AATCCTTTCTTTAATCTCAAATCA
22	AATCCTTTTTACTCAATTCAATCA
23	TTCAATCATTCAAATCTCAACTTT
24	TCAATTACCTTTTCAATACAATAC
25	CTTTTCAATTACTTCAAATCTTCA
26	TTACTCAAAATCTACACTTTTTCA
27	CTTTTCAAATCAATACTCAACTTT
28	CTACAAACAAACAAACATTATCAA
29	AATCTTACTACAAATCCTTTCTTT
30	TTACCTTTATACCTTTCTTTTTAC
31	TTCACTTTTCAATCAACTTTAATC
32	ATTATTCACTTCAAACTAATCTAC
33	TCAATTACTTCACTTTAATCCTTT
34	TCATTCATATACATACCAATTCAT
35	CAATTTCATCATTCATTCATTTCA
36	CAATTCATTTCATTCACAATCAAT
37	CTTTTCATCTTTTCATCTTTCAAT
38	TCAATCATTACACTTTTCAACAAT
39	TACACAATCTTTTCATTACATCAT
40	CTTTCTACATTATTCACAACATTA
41	TTACTACACAATATACTCATCAAT
42	CTATCTTCATATTTCACTATAAAC
43	CTTTCAATTACAATACTCATTACA
44	TCATTTACCAATCTTTCTTTATAC
45	TCATTTCACAATTCAATTACTCAA
46	TACATCAACAATTCATTCAATACA
47	CTTCTCATTAACTTACTTCATAAT
48	AAACAAACTTCACATCTCAATAAT
49	TCATCAATCTTTCAATTTACTTAC
50	CAATATACCAATATCATCATTTAC
51	TCATTTCAATCAATCATCAACAAT
52	TCAATCATCTTTATACTTCACAAT
53	TAATTATACATCTCATCTTCTACA
54	CTTTTTCAATCACTTTCAATTCAT
55	TATATACACTTCTCAATAACTAAC

56	CAATTTACTCATATACATCACTTT
57	CAATATCATCATCTTTATCATTAC
58	CTACTAATTCATTAACATTACTAC
59	TCATCAATCAATCTTTTTCACTTT
60	AATCTACAAATCCAATAATCTCAT
61	AATCTTACCAATTCATAATCTTCA
62	TCAATCATAATCTCATAATCCAAT
63	CTACTTCATATACTTTATACTACA
64	CTACATATTCAAATTACTACTTAC
65	CTTTTCATCAATAATCTTACCTTT
66	TAACATTACAACTATACTATCTAC
67	TCATTTACTCAACAATTACAAATC
68	TCATAATCTCAACAATCTTTCTTT
69	CTATAAACATATTACATTCACATC
70	ATACCAATAATCCAATTCATATCA
71	ATCATTACAATCCAATCAATTCAT
72	TCATTTACCTTTAATCCAATAATC
73	ATCAAATCTCATCAATTCAACAAT
74	TACACATCTTACAAACTAATTTCA
75	AATCATACCTTTCAATCTTTTACA
76	AATCTAACAAACTCATCTAAATAC
77	CAATTAACTACATACAATACATAC
78	CTATCTATCTAACTATCTATATCA
79	TTCATAACTACAATACATCATCAT
80	CTAACTAACAATAATCTAACTAAC
81	CTTTAATCTACACTTTCTAACAAT
82	TACATACACTAATAACATACTCAT
83	ATACAATCTAACTTCACTATTACA
84	TCAACTAACTAATCATCTATCAAT
85	ATACTACATCATAATCAAACATCA
86	CTAATTACTAACATCACTAACAAT
87	AAACTAACATCAATACTTACATCA
88	TTACTTCACTTTCTATTTACAATC
89	TATACTATCAACTCAACAACATAT
90	CTAAATACTTCACAATTCATCTAA
91	TTCATAACATCAATCATAACTTAC
92	CTATTACACTTTAAACATCAATAC
93	CTTTCTATTCATCTAAATACAAAC
94	CTTTCTATCTTTCTACTCAATAAT
95	TACACTTTAAACTTACTACACTAA
96	ATACTAACTCAACTAACTTTAAAC
97	AATCTCATAATCTACATACACTAT
98	AATCATACTCAACTAATCATTCAA
99	AATCTACACTAACAATTTCATAAC
100	CTATCTTTAAACTACAAATCTAAC

Supplementary Table 5 | barcode (sense) sequences

name	sequence	name	sequence
FW_NOTCH3	CCTAGACCTGGTGGACAAG	REV_NOTCH3	ACACAGTCGTAGCGGTTG
FW_JAG1	ACACACACTCAGCCTCTGAGGAC	REV_JAG1	GGGTTTTTGATCTGGTTCAGCT
FW_PIK3CA	ATCTTTTCTCAATGATGCTTGGCT	REV_PIK3CA	CTAGGGTCTTTCGAATGTATG
FW_TWIST	TCCGCGTCCCACTAGCA	REV_TWIST	TTCTCTGGAAACAATGACATCTAGGT
FW_CCNE1	GAGCCAGCCTTGGGACAATA	REV_CCNE1	CGGTCATCATCTTCTTTGTCAGG
FW_AKT1	CATCACACCACCTGACCAAG	REV_AKT1	CTGGCCGAGTAGGAGAACTG
FW_K-Ras	TAATTGATGGAGAAACCTGTCTCTTG	REV_K-Ras	TTATGGCAAATACACAAAGAAAGCC
FW_IKBKE	TGCGTGCAGAAGTATCAAGC	REV_IKBKE	TACAGGCAGCCACAGAACAG
FW_HDM2	TCTACAGGGACGCCATCGA	REV_HDM2	CTGATCCAACCAATCACCTGAA
FW_CCND1	GGATGCTGGAGGTCTGCGA	REV_CCND1	AGAGGCCACGAACATGCAAG
FW_SKP2	TCAACTACCTCCAACACCTATCAC	REV_SKP2	GACAACTGGGCTTTTGCAGT
FW_TP53	GCCCCCAGGGAGCACTA	REV_TP53	GGGAGAGGAGCTGGTGTTG
FW_ZNF217	TCTCAGAACGCATACAGGTGA	REV_ZNF217	CAGCAGCAACATCGGTTTGT
FW_HER2	AACTGCACCCACTCCTGTGT	REV_HER2	TGATGAGGATCCCAAAGACC
FW_WIP1	CCCATGTTCTACACCACCAGT	REV_WIP1	TGGTCCTTAGAATTCACCCTTG
FW_MDM2	TTAGTGGCTGTAAGTCAGCAAGA	REV_MDM2	CCTTCAGATCACTCCCACCT
FW_TWIST1	CGGGTCATGGCCAACGTG	REV_TWIST1	CAGCTTGCCATCTTGGAGTC
FW_mFGFR1	GCAGAGCATCAACTGGCTG	REV_mFGFR1	GGTCACGCAAGCGTAGAGG
FW_BRAF	CAAACTTATAGATATTGCACG	REV_BRAF	TCTGGTGCCATCCACAAAATG
FW_GSTM1	CTGGGCATGATCTGCTACAATC	REV_GSTM1	CAAAAGTGATCTTGTTTCCTGCAA
FW_cMYC	CTTCTCTCCGTCCTCGGATTCT	REV_cMYC	GAAGGTGATCCAGACTCTGACCTT
FW_GAPDH	CGAGCCACATCGCTCAGACA	REV_GAPDH	GGCGCCCAATACGACCAAAT

Supplementary Table 6 | qRT Primer sequences used

DRUG NAME	100x stock concentration (ng/µL)
(s)BI-2536 TsOH Salt	200
ABT-737	200
ABT-869	200
AC220	200
Actinomycin D	0.2
AG13958	20
Akt-I-1,2	200
Akt-I-1	2000
AMG-Tie2-1	20
AS-25242	200
AT7519	200
AT9283	2
AV-412	0.2
AZD6244	2
BEZ235	2
BI-D1870	2
BMS-2	200
BX795	20
BYK204165	20
CC-401	200
CI-1033	2
CI-1040	2
CP-690550	2000
CP-724714	200
CYC-116	2
CYT11387	2
Dasatinib	0.2
E7080	200
Erlotinib-HCl	2
Flavopiridol	0.2
Gefitinib	2
GW441756	200
HP-470	2000
Imatinib-Mesylate	200
JNJ-38877605	200
JNJ-7706621	2
KI20227	200
KU0063794	2
KU55933	200
Lapatinib	2
Masatinib	2
Merck-5	2
Nilotinib	20
Nocodazole	0.2
NU1025	2000
Pazopanib	20
PD0325910	2
PD-04217903	200
PD173955-Analogue 1	20

PD173955	200
PF431396	0.2
PF562271	2
PI103	2
PI93	20
PIK-75	0.2
PIK-90*	2
PLX4720	200
Purvalanol B	200
R1487	2000
Rho-15	200
RWJ-67657	200
SB202190	200
SB203580	200
SB216763	200
SB242235	200
SB590885	200
SNS-032	0.2
SNS-314	200
SR3677	200
SU-5402	200
SU6668	200
Sunitinib	20
TAK-715	200
Tandutinib	200
TG100115	200
TG101209 Deriv 1	2
TG101209 Deriv 2	2
TG101209	2
TG101348	20
TGX221	2000
Vandetanib	2
VX-680	2
VX-702	2000
YM201636	20
Z-LLL-AL	0.2
ZSTK474	2
DMSO	0.25%

Supplementary Table 7 | Selected compounds for the screen

drugName	regionName	beta	pvBeta
SNS-314_200nM	MYC_cDNA	-0.250292079	2.66E-14
AT9283_2nM	MYC_cDNA	-0.231393681	7.27E-13
BEZ235_2nM	NOTCH1_cDNA	0.178855423	1.08E-11
SNS-314_200nM	SMAD4_shRNA	-0.255918449	9.71E-11
AT9283_2nM	NOTCH1_cDNA	-0.166537633	1.37E-10
SNS-314_200nM	BRCA2_shRNA	-0.232039301	5.59E-09
BEZ235_2nM	BRCA2_shRNA	-0.219842682	2.57E-08
AT9283_2nM	JAG1_cDNA	-0.169850157	4.66E-08
PD0325910_2nM	NOTCH1_cDNA	-0.13575346	6.23E-08
SNS-314_200nM	PIK3CA_cDNA	-0.13307607	6.92E-08
TG101209_2nM	MDM2_cDNA	0.144766473	1.39E-07
TG101209_2nM	TWIST_cDNA	0.127552401	1.66E-07
Gefitinib_2nM	PIK3CA_cDNA	0.128199466	1.77E-07
Gefitinib_2nM	H-Ras_cDNA	0.158382488	1.87E-07
BMS-2_200nM	MYC_cDNA	-0.154180152	2.82E-07
SNS-314_200nM	NOTCH1_cDNA	-0.123306679	6.41E-07
SNS-314_200nM	WIP1_cDNA	-0.124335264	7.71E-07
AT9283_2nM	CCNE1_cDNA	-0.137270888	1.40E-06
Tandutinib_200nM	CADM1 = IGSF4_siRNA	-0.213285088	1.44E-06
Tandutinib_200nM	SKP2_cDNA	-0.151320891	2.23E-06
BEZ235_2nM	CDKN1B_shRNA	-0.253962065	2.74E-06
PDI7395_200nM	SKP2_cDNA	-0.14927083	2.96E-06
PDI7395_200nM	H-Ras_cDNA	-0.137905968	3.97E-06
SNS-314_200nM	H-Ras_cDNA	-0.137276229	4.35E-06
PDI7395_200nM	MYC_cDNA	-0.135154945	4.86E-06

Supplementary Table 8 | Top 25 hits from the screen.

2.1.2.1 *Supporting information*

- To mimic the mutational landscape of breast cancer with a great prudence we constructed an isogenic MCF10A cell line panel by viral transduction with single cDNAs or short hairpin RNAs (shRNAs). It is worth mentioning that the human breast epithelial cell line MCF10A is non-tumourigenic and known to be responsive to most signalling pathways found in normal breast epithelial cells (Muellner et al, 2011). It exhibits transcriptional features of both basal and luminal cell types (Muellner et al, 2011; Neve et al, 2006). Henceforth, MCF10A is thought to represent a multi-lineage progenitor cell line (Muellner et al, 2011; Neve et al, 2006). To investigate its suitability for our aim, we subjected MCF10A cells to a high density SNP array. The analysis unravelled various chromosomal alterations such as trisomies, amplifications and deletions (Figure 15) (Muellner et al, 2011). A previously documented homozygous deletion of the *INK4A* locus on chromosome 9 could also be affirmed by this analysis (Muellner et al, 2011) and was in agreement with qRT-PCR data (not shown) where *CDKN2A* gene expression was below detection levels (Iavarone & Massague, 1997). Nevertheless, compared to conventional breast cancer cell lines, MCF10A exhibited a relatively normal karyotype and therefore was selected as a model system (Muellner et al, 2011).

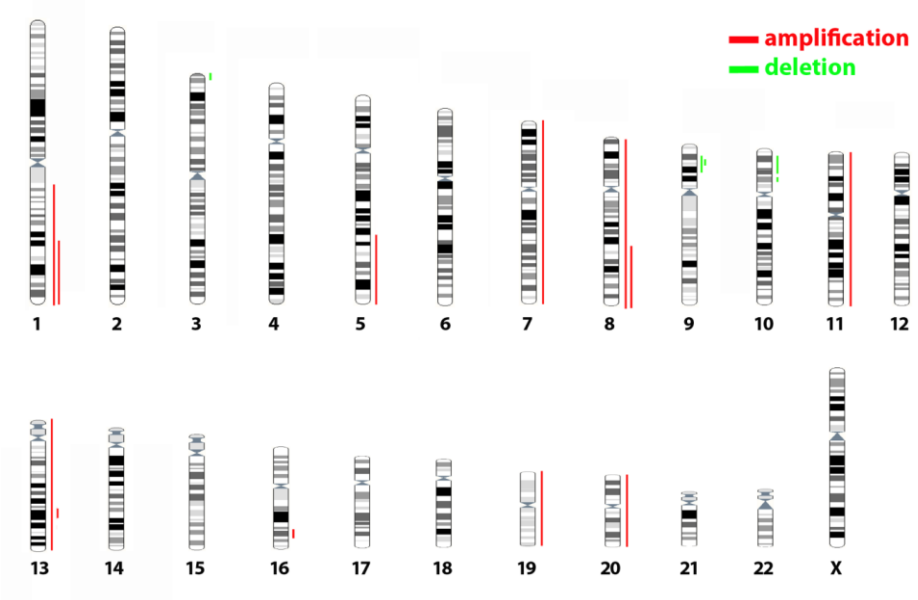


Figure 15. Karyoview of MCF10A cell line

Indicated are trisomies and amplifications in red and deletions in green.

- The isogenic MCF10A cell line panel capturing the genetic landscape of breast cancer was employed to screen for diverse FDA-approved and experimental anti-cancer drugs that attack cancer cells with specific mutations. Data was analysed using an ANOVA-based linear regression method (Muellner et al, 2011). The resulting dataset revealed numerous gene-compound interactions that could be confirmed in single well validation experiments. Among those are resistant interactions between gefitinib and constitutive active RAS^{V12} or PI3K catalytic subunit α-isoform (PIK3CA) (Table 11, Figure 16 a-b). This resistant phenotype to EGFR inhibition can be explained by the fact that both RAS and PI3K are downstream effectors of this tyrosine kinase receptor. In addition, the data analysis of the primary screen revealed resistance of TWIST cells to the JAK2 inhibitor TG101209 (Table 11). Strikingly, independent single well dose-response validation experiments unravelled the exact opposite phenotype (Figure 16 c). The enhanced sensitivity to JAK2 inhibition, however, can be explained by previous studies demonstrating the dependency of the $CD44^{+}/CD24^{-}$ breast cancer stem cell phenotype in human tumours on the overexpression of both TWIST and JAK2/STAT3 signalling (Marotta et al, 2011; Vesuna et al, 2009). Consequently, elimination of one of these two components provides the potential to strongly impair the tumour cell growth.

Compound	Gene alteration	Beta	pvBeta	Phenotype
TG101209_2nM	TWIST_cDNA	0.127552401	1.66E-07	Resistance
Gefitinib_2nM	PIK3CA_cDNA	0.128199466	1.77E-07	Resistance
Gefitinib_2nM	H-Ras_cDNA	0.158382488	1.87E-07	Resistance

Table 11. Selected hits from the MCF10A-based gene-compound screen

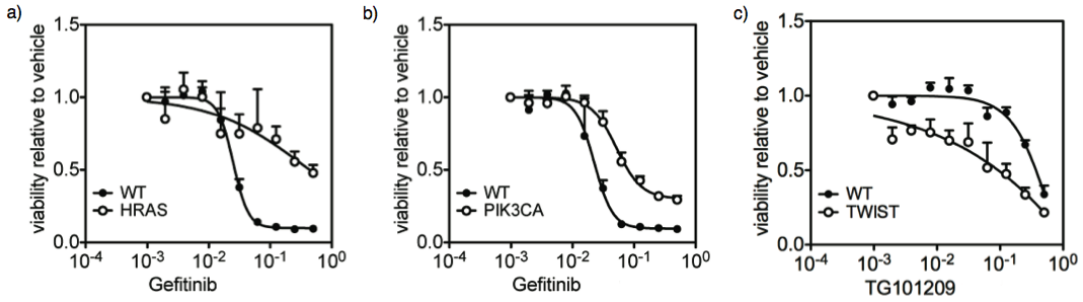


Figure 16. Validations of selected resistant or sensitivity interactions

(a-c) Dose-response analysis of indicated cells to EGFR inhibition (gefitinib) or JAK2 inhibition (TG101209), respectively. Wild type (WT) MCF10A cells were used as a control. Cells were treated with compounds for five days. Concentrations are log-transformed and given in ng/µl. Relative cell viability was assessed using CellTiter-Glo. Each graph is representative for at least four independent experiments performed in triplicate. Error bars indicate standard deviations.

2.1.3 Genetic synthetic lethality screens between cancer genes and druggable kinases

As an alternative to pharmacological screening, we took advantage of RNA interference (RNAi) technology and decided to expose the same isogenic MCF10A cell line panel to a commercial SMARTpool siRNA library (Figure 17 a). This collection consisted of 788 individual pools targeting all human protein kinases and few phosphatases (Dharmacon, referred to as kinome siRNA library) wherein each gene was silenced by a mixture of 4 siRNA duplexes (Table 25, page 180). Given that kinases are involved in basically all biological processes and represent readily accessible drug targets, a newly reported vulnerability through synthetic lethality in dependency on kinases would be directly useful in the clinic. Thus, we screened a total of 89 isogenic MCF10A cell lines against the kinome siRNA library at 50nM each in quadruplicate for seven days. As described in the section *2.1.2* (page 89) (Muellner et al, 2011), the approach is based on that each cell line also carries a unique DNA barcode sequence in its genome that allows screening in a pooled format, thus dramatically increasing throughput. In our assays barcodes were amplified from genomic DNA derived from the pool of genotypic variants by PCR, then fluorescently labeled and hybridized to microspheres that are coupled to the antisense barcodes. Subsequent analysis by flow-cytometry on a Luminex machine during which both the bead colour identity and associated fluorescence were

measured revealing the relative abundance of each barcode (Figure 17 a). The dataset was analysed on the impact on breast cancer survival using an ANOVA-based linear regression method as applied in the section *2.1.2* (page 89) (Muellner et al, 2011), which yielded tens of thousands pairwise measurements (Table 13 and data not shown).

In parallel, we wished to investigate whether potential synthetic lethalities are conserved among different cell types. The reasoning behind this can be explained as follows: First of all, a significant fraction of synthetic lethal interactions has been reported to be conserved between *S. cerevisiae* and *S. pombe,* two organisms that are 300-600 million years apart in evolution (Dixon et al, 2008; Nijman, 2011; Roguev et al, 2008). Moreover, recent studies highlighted highly conserved transcriptional networks between tissues and organisms (Lamb et al, 2006). Microarray analysis of the NCI60 cell lines further supports that most tissues express the majority of the cancer-relevant genes. Finally, high-throughput screens for anti-cancer compounds unravelled clinically relevant, conserved vulnerabilities among cancer cells (Barretina et al, 2012; Garnett et al, 2012). All these strongly support the hypothesis that different tumour types will share at least a proportion of the synthetic lethalities. For this purpose, we decided to use an isogenic cell line system based on HeLa cells (cervix carcinoma). We infected cells only with barcoded lentiviral vectors expressing a distinct shRNA against one of the well-known 38 tumour suppressor genes under study, yielding a total of 80 isogenic cell lines (Table 12). The majority of knock-downs were confirmed by immunoblotting and qRT-PCR (data not shown). These tumour suppressors are frequently found deleted or otherwise inactivated in a broad variety of tumours, many of them being breast-cancer relevant, and the mechanisms and pathways affected are diverse. It is worth remembering that directly targeting loss-of-function mutations, such as those found in tumour suppressor genes, with drugs remain laborious. Yet, some may be indirectly attacked by exploiting synthetic lethal interactions. Thus, the pooled cell line panel was subsequently employed to screen for the kinome siRNA library at 50nM each for seven days as described above (Figure 17 b), where we noted around thirty thousand gene-gene interaction pairs (Table 13 and data not shown). Validation experiments of the highest scoring synthetic lethality hits from both MCF10A and HeLa genetic screens, however, did not confirm the observed sensitivity to corresponding kinase knock-downs. The potential reasons behind these discrepancies will be discussed in the section *3.1* (page 153).

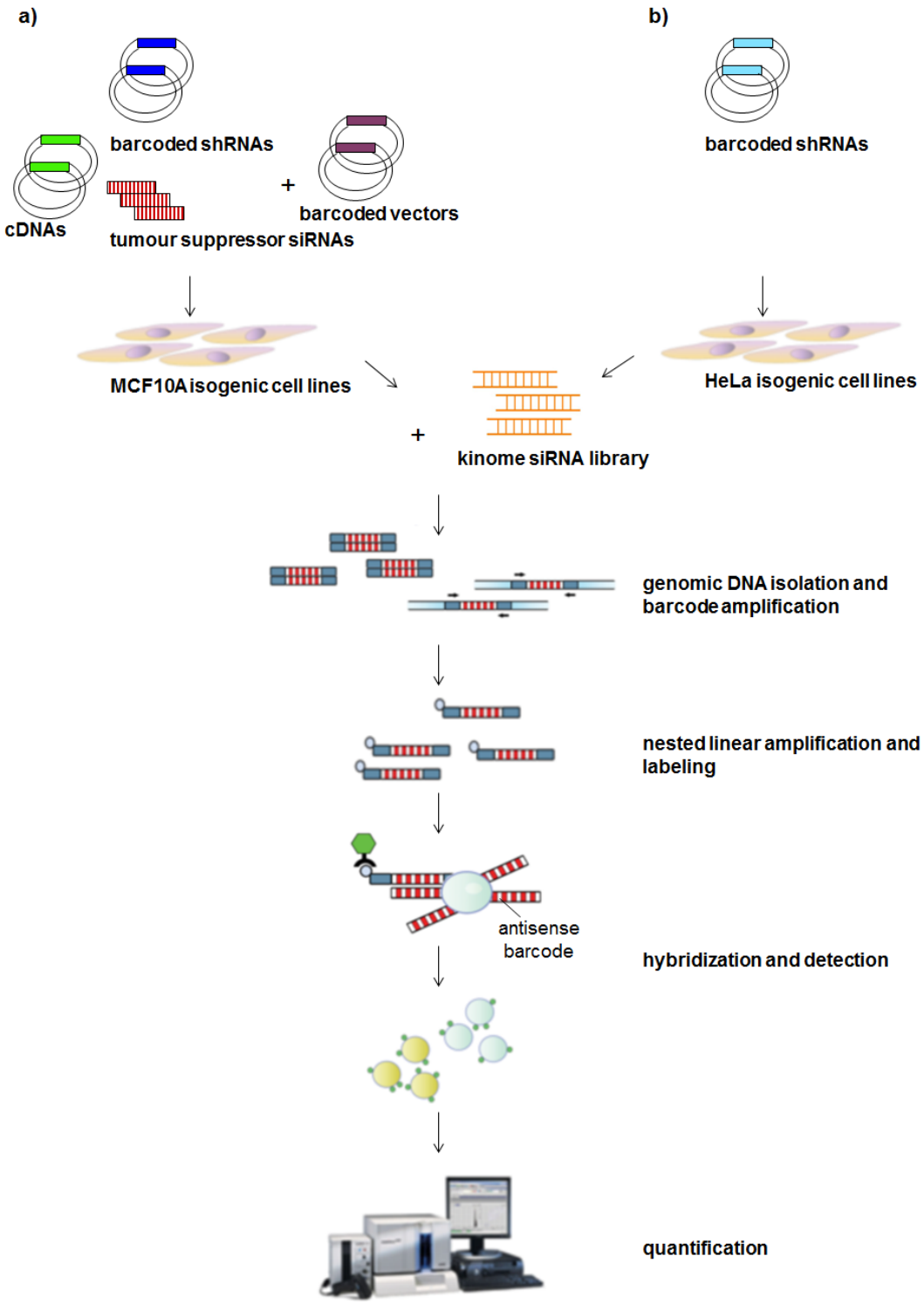


Figure 17. Gene-gene synthetic lethality screening strategy

MCF10A- (a) or HeLa (b)-based isogenic cell lines, each harbouring a cancer-driving alteration, were pooled together, seeded into multiple 96-well plates and subjected to a kinome siRNA library. After genomic DNA isolation, barcodes, each representative for the abundance of a certain cell line, were amplified by PCR and fluorescently labelled by a second nested linear amplification using a biotinylated primer. Following hybridization to Luminex xMAP beads, the amplification products were quantified by flow cytometry on a Luminex machine. Isogenic cells found more sensitive upon exposure to a particular kinase siRNA were validated in independent experiments.

APC	Adenomatosis polyposis coli
ATM	Ataxia telangiectasia mutated
BECN1	Beclin 1
BRCA1	Breast cancer 1, early onset
BRCA2	Breast cancer 2, early onset
CDC73	Cell division cycle 73
CDKN1B	Cyclin-dependent kinase inhibitor 1B, p27
CDKN1C	Cyclin-dependent kinase inhibitor 1C, p57
CDKN2A	Cyclin-dependent kinase inhibitor 2A, p16
CDKN2A	p14-ARF
CHEK2	Serine/threonine-protein kinase Chk2
CYLD	Cylindromatosis gene
DAPK1	Death-associated protein kinase 1
FAT2	FAT tumour suppressor homologue 2
FAT3	FAT tumour suppressor homologue 3
FH	Fumarate hydratase
FHIT	Fragile histidine triad protein
FLCN	Folliculin
FOXP3	Forkhead box P3
KLF6	Krüppel like factor 6
MEN1	Multiple endocrine neoplasia 1
NBN	Nibrin, NBS1
NF1	Neurofibromin 1
NF2	Neurofibromin 2
NOTCH1	Notch homologue 1
PPP2R1B	Protein phosphatase 2 subunit A (PR65)
PTCH1	Patched homologue 1
PTEN	Phosphatase and tensin homologue
RB1	Retinoblastoma protein 1
SDHD	Succinate dehydrogenase subunit D
SMAD2	SMAD family member 2
SMAD4	SMAD family member 4
STK11	Serine/threonine kinase 11, LKB1
TP53	Tumour protein p53
TSC1	Tuberous sclerosis 1
TSC2	Tuberous sclerosis 2
VHL	Von Hippel-Lindau tumour suppressor
WT1	Wilms tumour 1
WTX	Wilms tumour X-associated

Table 12. Tumour suppressor genes used for shRNA knock-down in HeLa cells

Cancer gene alteration	Kinase siRNA treatment
IKBE_cDNA	MULK
RASSF1_siRNA	JNK2
RASSF1_siRNA	ERK2
APC_shRNA	MARK1
APC_shRNA	MAPK13
BECN1_shRNA	CDK4
p57_shRNA	EIF2AK3
p27_shRNA	MLCK
BECN1_shRNA	CCL4
NF2_shRNA	KIAA1804
STK11_shRNA	MAP3K12
FBXW7_shRNA	KIT
VHL_shRNA	MARK1
p27_shRNA	ERK8
WTX_shRNA	MATK
CHEK2_shRNA	NEK5
NBN_shRNA	MAPK6
TP53_shRNA	CCRK
WT1_shRNA	CLK4
CDC73_shRNA	STYK1

Table 13. Top 20 hits from gene-gene synthetic lethality screen

2.2 USP4 regulates PDK1 mono-ubiquitination

2.2.1 Prologue

Breast-cancer driving genetic abnormalities have recently been linked to only a small number of signalling pathways, highly suggesting that attacking pathways as cancer Achilles' heels rather than individual genes may be more robust and effective and as a consequence, improve the treatment outcome (Curtis et al, 2012; Shah et al, 2012). Among such pathways that frequently contribute to breast cancer susceptibility is the PI3K-PDK1-AKT signalling cascade that is associated with poor prognosis. In particular, one in five breast cancer patient amplifies or overexpresses the protein kinase PDK1 (Liu et al, 2009; Rodon et al, 2013). Hence, this signalling presents an attractive therapeutic target. Strikingly, to date, how PDK1 activity is controlled remains unclear and consequently, no approved drugs specifically targeting PDK1 are available.

Henceforth, as stated in the section *1.4.3* (Aims of the study, page 86), the purpose of this work was to survey for novel druggable modulators of PDK1 and its downstream signalling. This would help us to advance our knowledge on PDK1 regulation and may hold the great potential to provide a novel targeting angle to optimize patient response. This aim was addressed by screening the impact of the ubiquitination and deubiquitination machinery on PDK1 and its downstream signalling.

2.2.2 Manuscript

Uras IZ et al. Ubiquitin-specific protease 4 inhibits mono-ubiquitination of the master growth factor signaling kinase PDK1 (PLOS ONE. 2012;7(2):e31003. doi: 10.1371/journal.pone.0031003) (Uras et al, 2012).

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Ubiquitin-Specific Protease 4 Inhibits Mono-Ubiquitination of the Master Growth Factor Signaling Kinase PDK1

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Abstract

Background: Phosphorylation by the phospho-inositide-dependent kinase 1 (PDK1) is essential for many growth factor-activated kinases and thus plays a critical role in various processes such as cell proliferation and metabolism. However, the mechanisms that control PDK1 have not been fully explored and this is of great importance as interfering with PDK1 signaling may be useful to treat diseases, including cancer and diabetes.

Methodology/Principal Findings: In human cells, few mono-ubiquitinated proteins have been described but in all cases this post-translational modification has a key regulatory function. Unexpectedly, we find that PDK1 is mono-ubiquitinated in a variety of human cell lines, indicating that PDK1 ubiquitination is a common and regulated process. Ubiquitination occurs in the kinase domain of PDK1 yet is independent of its kinase activity. By screening a library of ubiquitin proteases, we further identify the Ubiquitin-Specific Protease 4 (USP4) as an enzyme that removes ubiquitin from PDK1 *in vivo* and *in vitro* and co-localizes with PDK1 at the plasma membrane when the two proteins are overexpressed, indicating direct deubiquitination.

Conclusions: The regulated mono-ubiquitination of PDK1 provides an unanticipated layer of complexity in this central signaling network and offers potential novel avenues for drug discovery.

Citation: Uras IZ, List T, Nijman SMB (2012) Ubiquitin-Specific Protease 4 Inhibits Mono-Ubiquitination of the Master Growth Factor Signaling Kinase PDK1. PLoS ONE 7(2): e31003. doi:10.1371/journal.pone.0031003

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Introduction

Growth factors such as insulin promote fundamental cellular processes such as proliferation and survival through the activation of signaling cascades involving numerous protein kinases. Of these, the phosphoinositide-3 kinase (PI3K) and the phosphoinositide-dependent kinase 1 (PDK1) play a critical role [1]. Upon activation, PI3K generates second messenger molecules consisting of phosphoinositides that prime members of the AGC superfamily of protein kinases for activation. At least twenty-three of the AGC kinases require phosphorylation by PDK1 at a conserved residue in their activation loop (also called T-loop), including central enzymes such as the proto-oncogene AKT, Protein Kinase C (PKC) and the p70 S6 kinase (S6K) [2,3,4,5]. Together, these and other downstream kinases coordinate cell growth, proliferation, survival and metabolism and they are frequently found deregulated in many diseases such as cancer and diabetes [6,7,8,9].

The mechanisms that keep PDK1 activity in check are not fully investigated. Yet, these are of great interest as the ability to interfere with the activation of its target kinases would be of great therapeutic importance. Given that PDK1 is such an important kinase, it is remarkable that it is found constitutively active due to *in trans* autophosphorylation of its T-loop residue [10]. Indeed, regulation of substrate accessibility is thought to be a major mechanism whereby PDK1 activity is controlled. In the case of AKT, this is achieved by recruitment to phospholipids generated by PI3K at the plasma membrane via the Pleckstrin Homology (PH) domains of both kinases. For other AGC kinases such as S6K and serum- and glucocorticoid-induced kinase (SGK), a priming phosphorylation on the hydrophobic motif by other kinases stimulates interaction with the PDK1 interacting fragment (PIF) pocket near the catalytic domain of PDK1, thereby facilitating T-loop phosphorylation [5,11,12]. PDK1 has also been described to shuttle between the nucleus and the cytoplasm in a growth factor dependent manner but the significance of this in terms of target activation has not been addressed [13,14]. Given the central role of PDK1 in the regulation of many downstream effectors, it is likely that additional regulatory mechanisms remain to be discovered.

The addition of the small molecule ubiquitin to proteins plays a critical role in essentially all biological processes. Indeed, defects in this control mechanism can cause many diseases including cancer [15,16,17]. The addition of polyubiquitin chains to proteins was originally identified as a mechanism for targeting

proteins to the 26S proteasome for degradation. However, it has recently been shown that selective mono-ubiquitination or alternative ubiquitin chains can also regulate protein activity [18,19,20,21,22,23,24]. These non-proteolytic functions of ubiquitination play diverse roles in DNA damage repair, protein trafficking and localization and activation of signal transduction pathways. Like phosphorylation, ubiquitination is reversible and mediated by deubiquitinating enzymes (DUBs) that cleave the isopeptide bond at the carboxy terminus of ubiquitin [25]. Furthermore, DUBs have also become actively studied drug targets for cancer therapy [26,27].

Various PI3K pathway components are regulated by ubiquitination, including AKT and PTEN [28,29,30]. However, for PDK1 no post-translational modification other than phosphorylation has been described. Here, we show that PDK1 is mono-ubiquitinated and that this modification occurs in the amino-terminal kinase domain. In addition, a cDNA library consisting of DUBs was screened for novel regulators of PDK1 ubiquitination and USP4 was identified as a potential modulator of PDK1 ubiquitination.

Results

PDK1 is modified by mono-ubiquitination

To investigate if PDK1 is ubiquitinated, we isolated ubiquitin-modified proteins from HEK293T cells by His-tagged ubiquitin pull-down under denaturing conditions (Figure 1A). Using a PDK1 specific antibody, we observed several distinct bands of which the most abundant migrated approximately 5–10 kDa higher than the major PDK1 isoform. This suggested that PDK1 is modified by a single ubiquitin moiety and the presence of multiple bands is in agreement with previous reports concerning PDK1 splice isoforms [31]. As mono-ubiquitination generally affects protein functionality and therefore may play a role in PDK1 regulation, we wished to explore this observation further. We henceforth refer to the ubiquitin-modified form of PDK1 as Ub-PDK1.

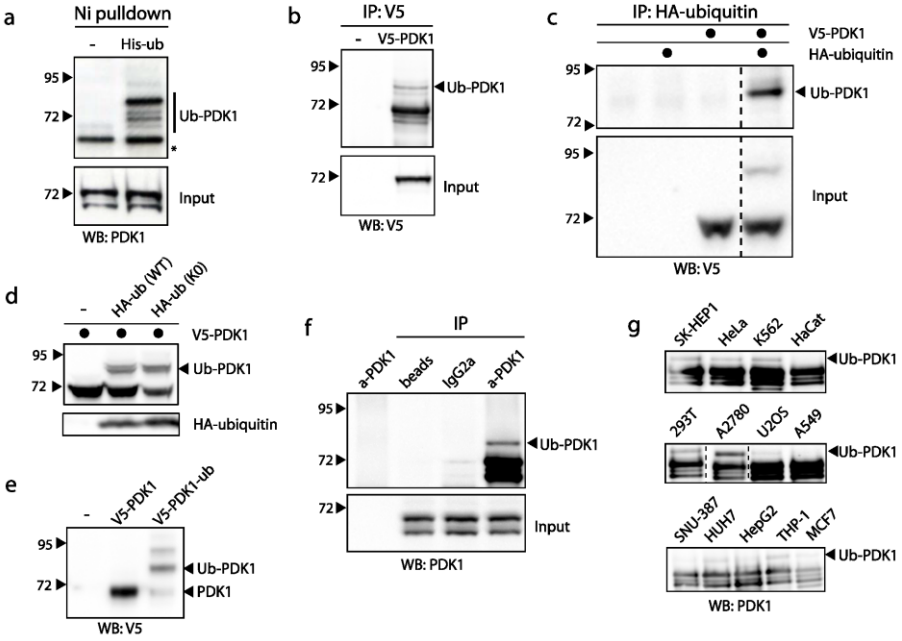

Figure 1. PDK1 is mono-ubiquitinated in a variety of cell lines. A: HEK293T cells were transfected with 6×His-tagged ubiquitin and ubiquitinated proteins were isolated with nickel beads under denaturing conditions. Pull-downs and input were analyzed with an antibody recognizing PDK1. Ub-PDK1 indicates ubiquitin-modified PDK1 species; asterisk indicates unspecific cross-reacting band. **B**: HEK293T cells were transfected with a V5-tagged PDK1 cDNA and V5-PDK1 was immunoprecipitated. Immunoprecipitations (IP) and input were immunoblotted with a V5 antibody. **C**: HEK293T cells were co-transfected as indicated with HA-tagged ubiquitin and V5-PDK1. Immunoprecipitation of HA-ubiquitin and input were immunoblotted with a V5 antibody. **D**: HEK293T cells were co-transfected as indicated and whole cell extracts were probed with anti-V5 or anti-HA antibody. **E**: HEK293T cells were transfected with V5-tagged PDK1 or a linear PDK1-ubiquitin C-terminal fusion protein. The whole cell extracts were probed with anti-V5 antibody. **F**: Endogenous PDK1 was immunoprecipitated from HEK293T cells and blotted with anti-PDK1 antibody. The following controls were used: anti-PDK1 antibody only, sepharose beads only, anti-IgG2a control antibody with beads. **G**: Lysates from the indicated cell lines were subjected to PDK1 immunoprecipitation. Immunoprecipitations were immunoblotted with a PDK1 antibody.
doi:10.1371/journal.pone.0031003.g001

To definitively characterize the observed band as mono-ubiquitinated PDK1, we first expressed V5-tagged PDK1 and performed V5 immunoprecipitation. As expected, immunoblotting with an anti-V5 antibody revealed several PDK1 bands, one of which migrated at the predicted molecular weight of mono-ubiquitinated PDK1 (Figure 1B). Co-expression of HA-tagged ubiquitin and V5-PDK1 further enhanced ubiquitination such that Ub-PDK1 became visible in the whole cell extract (Figure 1C, lower panel) and anti-HA immunoprecipitation confirmed that the slower migrating PDK1 band corresponds to Ub-PDK1 (Figure 1C, upper panel). Furthermore, co-expression of a lysine-less ubiquitin mutant (K0) that cannot mediate chain formation resulted in a similar Ub-PDK1 pattern, further indicating that the observed band is due to a single ubiquitin moiety (Figure 1D). In agreement with this, a linear PDK1-ubiquitin C-terminal fusion protein migrated at the same position in the gel as Ub-PDK1 (Figure 1E).

To determine whether we could detect endogenous Ub-PDK1, we performed PDK1 immunoprecipitation experiments. Immunoblotting with an anti-PDK1 antibody revealed a clear, albeit minor Ub-PDK1 band at the predicted molecular weight (Figure 1F). Importantly, varying levels of endogenous Ub-PDK1 were observed in a variety of cell lines derived from different tumor types (Figure 1G). Together, these experiments indicate that PDK1 mono-ubiquitination is a common and differentially regulated post-translational modification.

Mono-ubiquitination of PDK1 occurs in the kinase domain

To further characterize this novel post-translational modification, we analyzed its site of attachment using a PDK1 deletion mutant (Figure 2A). Cells transfected with full-length PDK1 or just the kinase domain, were analyzed for Ub-PDK1 (a PDK1 mutant lacking the kinase domain was not stably expressed and thus not included in this experiment). A band migrating approximately 5–10 kDa higher than the kinase domain was detectable, suggesting that this domain is ubiquitinated (Figure 2B). Indeed, this was confirmed to be ubiquitinated PDK1 by HA-ubiquitin immunoblot (Figure 2C). In this experiment we detected a second Ub-PDK1 band (labeled Ub-PDK1$_{(2)}$) migrating some 5–10 kDa above the mono-ubiquitinated PDK1, indicating conjugation of two ubiquitin moieties onto a single PDK1 molecule. However, we did not detect conjugation of multiple ubiquitin polypeptides to endogenous PDK1 in the absence of exogenous ubiquitin, suggesting that this only occurs upon overexpression (Figure 1F).

To exclude the possibility that ubiquitin can also conjugate to the PH domain and to further corroborate the observation that ubiquitination occurs in the kinase domain, we mutated all 27

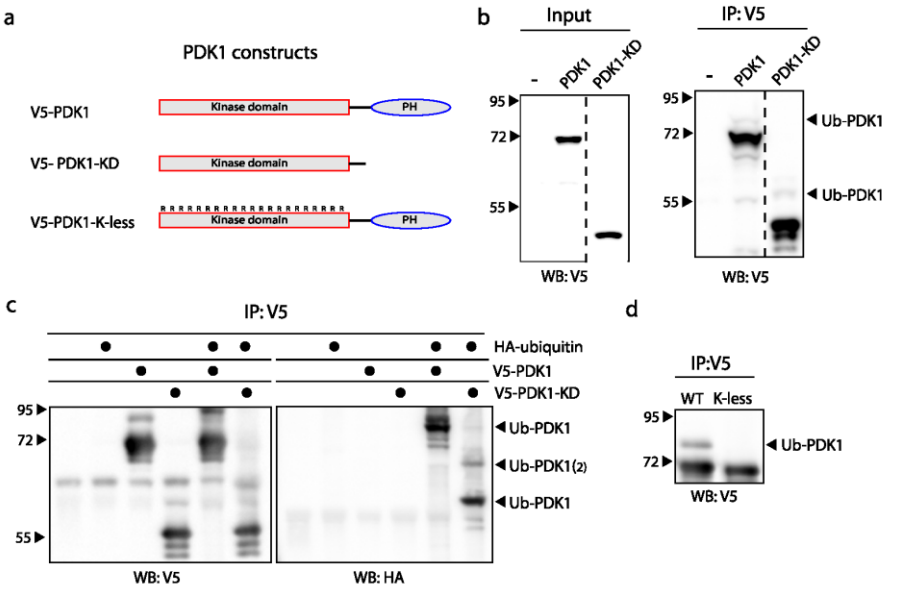

Figure 2. The kinase domain of PDK1 is mono-ubiquitinated. A: Overview of the PDK1 constructs used in the figure. PDK1 consists of an amino-terminal kinase domain (KD) and a carboxyl-terminal Pleckstrin Homology (PH) domain. The K-less mutant has all 27 lysine residues (K) mutated to arginines (R). **B**: HEK293T cells were transfected as indicated and PDK1 was immunoprecipitated with anti-V5 beads. Immunoprecipitations (IP) and input were immunoblotted with anti-V5 antibody. **C**: HEK293T cells were transfected as indicated and proteins were immunoprecipitated with anti-V5 beads. Immunoprecipitations (IP) were immunoblotted with V5 and HA antibodies. **D**: HEK293T cells were transfected and the lysine-less mutant of PDK1 was immunoprecipitated with anti-V5 beads and probed with anti-V5 antibody. The wild type construct (WT, V5-PDK1) was used as a control.
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lysine (K) residues of the kinase domain into arginines (R) while keeping the PH domain intact (Figure 2A). As expected, this K-less mutant was not mono-ubiquitinated (Figure 2D). Thus, ubiquitination of PDK1 occurs in the kinase domain.

Evolutionary conserved lysine residues are not ubiquitinated

PDK1 comprises 38 lysine residues, each of which could potentially form an isopeptide bond with an ubiquitin molecule. Unfortunately, mutating back each single arginine residue in the K-less mutant to lysine did not restore mono-ubiquitination (not shown). Next, we mutated twelve of the lysine residues conserved between worms, fruit fly, mouse and humans to investigate if any of these would be required for PDK1 mono-ubiquitination. Mutation of K441 and K492/494 resulted in reduced PDK1 protein expression when compared to the GFP transfection control, explaining the reduction in Ub-PDK1 in these samples and indicating that these lysines are not the principal conjugation residues (Figure 3A). Also mutation of the other conserved lysines did not result in a dramatic change of Ub-PDK1. Furthermore, mass spectrometry analysis of purified and trypsin digested Ub-PDK1 was unable to identify the target residue, despite detection of the great majority of predicted tryptic peptides (not shown). A potential explanation for these results may be that PDK1 can be ubiquitinated on various redundant lysine residues, as has been shown for AKT, p53 and Cyclin B, thereby making both the genetic and biochemical approach to identify the site(s) highly challenging [28,32,33].

Ub-PDK1 is nuclear and cytoplasmic, binds phospholipids and mono-ubiquitination is not dependent on kinase activity

PDK1 shuttles between the nucleus and the cytoplasm in a CRM1-dependent and growth factor regulated manner but the functional significance of this remains unclear [13,14]. As mono-ubiquitination has been reported to regulate subcellular localization, we investigated a potential role for PDK1 mono-ubiquitination in this process. Cells were transfected with PDK1 to enable detection of mono-ubiquitinated PDK1 by immunoblotting, and subsequently fractionated into nuclear, cytoplasmic and membrane fractions. Ub-PDK1 was found to be equally distributed over the nuclear, cytoplasmic and membrane fractions (Figure 3B).

As mentioned, PDK1 is recruited to the plasma membrane via its association with phospholipids such as PIP3, where it is known to function in phosphorylating target proteins. To determine whether PDK1 mono-ubiquitination plays a role in binding to the plasma membrane, we incubated cell lysates from cells transfected with V5-tagged PDK1 with phospholipid-coated beads. The ratio PDK1:Ub-PDK1 in the input and lipid bead bound fraction was very similar (compare lanes 2 and 5), indicating that Ub-PDK1 binds equally well to phospholipid-coated beads as unmodified PDK1. Furthermore, a PDK1 PH domain point mutant that impairs phospholipid binding [34] was still mono-ubiquitinated, indicating that lipid binding is not a pre-requisite for ubiquitination (Figure 3C). Together, these observations indicate that PDK1 mono-ubiquitination is not involved in membrane binding, at least under these conditions.

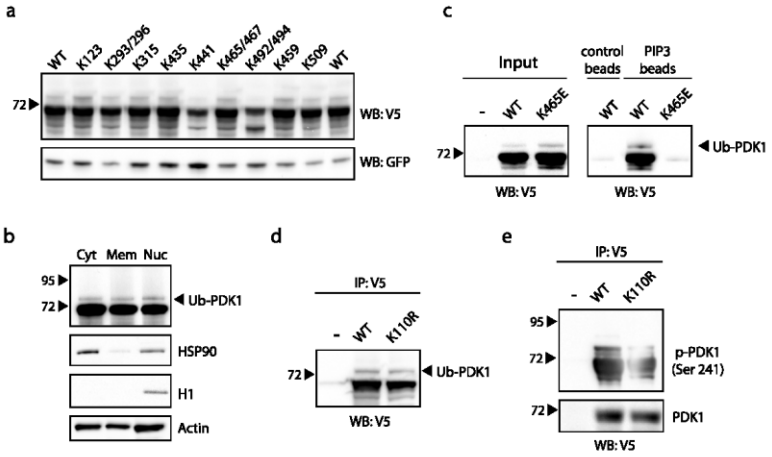

Figure 3. Ub-PDK1 localizes to all cellular compartments, binds phospholipids and mono-ubiquitination is not dependent on kinase activity. A: HEK293T cells were co-transfected with wild type V5-PDK1 (WT) or the indicated lysine mutants and GFP. Whole cell extracts were blotted and probed with anti-V5 and GFP that served as a transfection efficiency control. **B**: Cell fractionation analysis of HEK293T cells transfected with V5-tagged PDK1 (Cyt = cytoplasmic, Mem = membrane bound, Nuc = nuclear). Ub-PDK1 was detected with a V5 antibody. The HSP90, Histone H1 (H1) and Actin antibodies were used as controls. **C**: HEK293T cells were transfected as indicated and PDK1 was pulled down using phospholipid-coated (PIP3) or control beads. Pull-downs and input were probed with anti-V5 antibody. **D**: HEK293T cells were transfected and the catalytically inactive mutant K110R of PDK1 was immunoprecipitated with anti-V5 beads and probed with anti-V5 antibody. The wild type construct (WT, V5-PDK1) was used as a control. **E**: HEK293T cells were transfected as indicated and PDK1 was immunoprecipitated with anti-V5 beads. Immunoprecipitations (IP) were analyzed with an antibody recognizing phosphorylated PDK1 at the Ser241 site.
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Next, we determined whether PDK1 mono-ubiquitination is dependent on its kinase activity. A catalytically inactive PDK1 K110R point mutant [35] was found to be comparably mono-ubiquitinated as wild type PDK1 and analysis with a phospho-specific antibody showed that both PDK1 species were phosphorylated, indicating that kinase activity is not required for PDK1 mono-ubiquitination (Figure 3D and 3E).

USP4 deubiquitinates PDK1 and co-localizes at the plasma membrane

As mono-ubiquitination does not lead to protein degradation and can be reverted by deubiquitinating enzymes (DUBs), we screened a total of 70 DUBs for their ability to inhibit Ub-PDK1 upon overexpression (Figure 4A). Tagged PDK1 and each individual DUB were co-expressed in HEK293T cells and analyzed for Ub-PDK1. Although the great majority of DUBs was expressed at high levels (Figure S1), only the Ubiquitin-Specific Protease 4 (USP4) reproducibly inhibited Ub-PDK1 (Figure 4A and 4B). Other candidate hits from the screen, including #24 and #56, failed to show a reproducible reduction in Ub-PDK1 (Figure S2). A catalytically inactive USP4 mutant (C311S), in which the active site cysteine 311 residue is mutated to serine, failed to modulate PDK1 ubiquitination, indicating that the effect of USP4 on PDK1 is dependent on its deubiquitinase activity (Figure 4B). Importantly, the inhibitory effect of USP4 on Ub-PDK1 could also be shown for endogenous PDK1 (Figure 4C and 4D). In addition, the closely related USP15, which shares 61% amino acid sequence identity with USP4 [36], had no effect on Ub-PDK1 levels, further suggesting that the ability of USP4 to deubiquitinate PDK1 is specific.

To investigate if the effect of USP4 on reducing PDK1 ubiquitination could be mediated by direct deubiquitination, we asked if USP4 displayed *in vitro* activity towards Ub-PDK1. Wild type or catalytically inactive USP4 were immunoprecipitated from transfected cells and incubated with purified PDK1/Ub-PDK1. A marked reduction in Ub-PDK1 was evident only in the sample incubated with wild type USP4, indicating that Ub-PDK1 serves as a direct substrate (Figure 4E).

To further investigate a potential role of USP4 in PDK1 deubiquitination, we asked if the two proteins interact upon overexpression. As predicted, MYC-tagged USP4 could be co-immunoprecipitated with V5-PDK1 in HEK293T cells (Figure 5A). A direct effect of USP4 on PDK1 was also suggested by co-localization studies using confocal microscopy. In accordance with previous studies, PDK1 was found to be mainly cytoplasmic with some additional nuclear staining [13,37]. Interestingly, USP4 and PDK1 co-localized intensely at the plasma membrane when the two proteins were overexpressed (Figure 5B). Taken together, these results identify USP4 as a putative regulator of Ub-PDK1.

Discussion

We identify a novel post-translational modification, mono-ubiquitination, of PDK1, which plays a central role in signaling via the PI3K pathway to control many cellular processes. We did not obtain evidence that PDK1 ubiquitination is regulated by growth factor signaling. For instance, total Ub-PDK1 did not change upon stimulation with insulin, EGF or glucose under the conditions we tested (not shown). Also cellular fractionation did not reveal a compartment-specific response upon stimulation with insulin (not shown). Nonetheless, it is plausible that PDK1 ubiquitination is modulated under specific conditions or by certain stimuli. Indeed, there is ample evidence that mono-ubiquitination is known to play a crucial role in protein function. For instance, upon DNA damage, the Fanconi Anemia protein FANCD2 is mono-ubiquitinated and re-localizes to the chromatin whereas ubiquitin-modified proliferating cell nuclear antigen (PCNA) recruits a specific DNA polymerase [38,39].

Furthermore, mono-ubiquitination of tyrosine kinase receptors triggers receptor endocytosis and p53 mono-ubiquitination stimulates its nuclear export [40,41]. Indeed, future studies will undoubtedly reveal a key regulatory function of PDK1 mono-ubiquitination. This function will be of particular interest as it is not fully understood how PDK1 orchestrates the activation of its many substrates [42]. For instance, PDK1 ubiquitination may modulate binding to adapter molecules and downstream targets or alter subcellular localization. However, the unavailability of a PDK1 point mutant that is not ubiquitinated hampered the investigation of these hypotheses. Indeed, reintroducing single lysine residues in the lysine-less PDK1 mutant did not restore mono-ubiquitination perhaps because mutation of all lysines in the kinase domain precludes interaction with the responsible ubiquitin ligase. The lack of success of the reverse experiment in which conserved lysines were mutated to arginines may be explained by redundancy between target lysines. Perhaps future mass spectrometry experiments with alternative proteases will reveal the ubiquitination site(s) on PDK1 and will enable a systematic functional analysis.

By screening the great majority of DUBs in the human genome, we identified USP4 as the only DUB enzyme that reduced PDK1 ubiquitination. This high degree of specificity makes USP4 a prime candidate as a negative regulator of Ub-PDK1. USP4 has previously been implicated in a number of processes, including protein quality control in the endoplasmatic reticulum and p53 and Wnt signaling [43,44,45,46]. Interestingly, USP4 has been reported to inhibit the kinase TAK1 that is ubiquitinated by the AKT regulator TRAF6. Thus, USP4 may impinge on the PI3K/PDK1/AKT pathway at multiple levels. However, we could not show effects on Ub-PDK1 upon knockdown of USP4, which is possibly explained by functional redundancy with other DUBs or low basal activity of endogenous USP4 (not shown).

In summary, we identified mono-ubiquitination as a novel post-translational modification of PDK1 and propose USP4 as a candidate negative regulator, adding an additional layer of complexity to the PDK1 signaling network.

Materials and Methods

Cell lines

The human cell lines were obtained from the American Type Culture Collection. HEK293T (human embryonic kidney cells), HeLa (cervix carcinoma), U2OS (osteosarcoma), HaCat (keratinocytes), A549 (alveolar basal epithelial carcinoma), MCF7 (breast adenocarcinoma), SK-HEP1 and HUH7 (hepatoma) cells were maintained in Dulbecco's modified Eagle's medium. K562 (chronic myelogenous leukemia), A2780 (ovarian carcinoma), THP-1 (acute monocytic leukemia), SNU-387 and HepG2 (hepatoma) were cultured in RPMI-1640 medium. All media were supplemented with 10% fetal calf serum and 1% penicillin/streptomycin at 37°C, 5% CO_2 and 95% humidity.

Immunoprecipitation, pull-downs and immunoblotting

For immunoprecipitation and PIP3 (Echelon Biosciences) pull-down, cell extracts were prepared in RIPA buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Tergitol, 0.5% Triton X-100 and 0.1% SDS) or in ELB buffer (0.1% NP-40, 50 mM Hepes, 250 mM NaCl and 5 mM EDTA) supplemented with protease

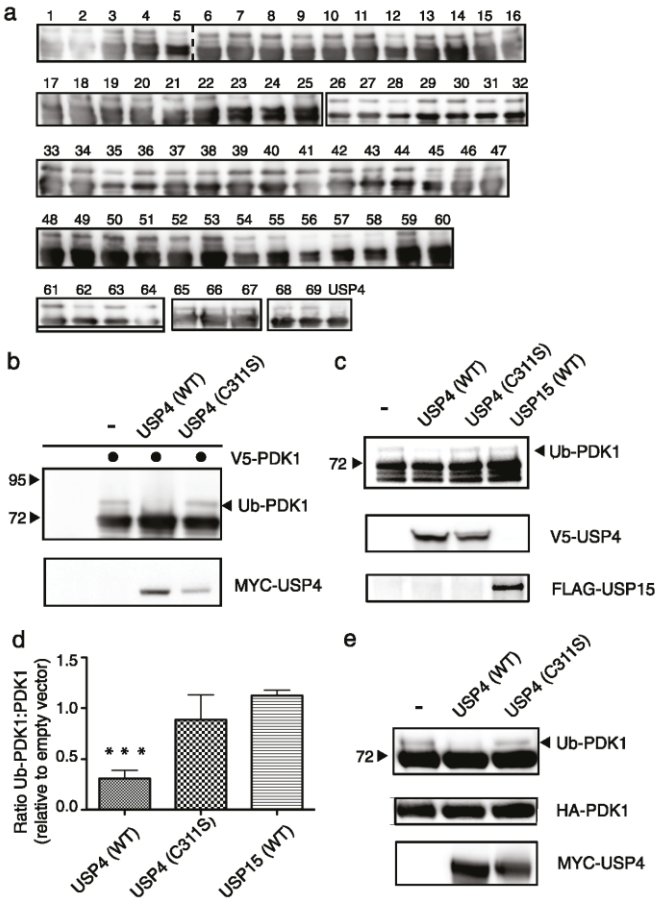

Figure 4. USP4 deubiquitinates PDK1 *in vivo* and *in vitro*. **A**: HEK293T cells were co-transfected with V5-tagged PDK1 and a DUB cDNA library. PDK1 was immunoprecipitated with anti-V5 beads and the Ub-PDK1 was detected using a V5 antibody. **B**: HEK293T cells were co-transfected as indicated. Proteins were immunoprecipitated with anti-V5 beads and immunoblotted with anti-V5 antibody. Whole cell lysates were probed with a MYC antibody to verify the expression of USP4 constructs. **C**: HEK293T cells were transfected with indicated DUBs. Endogenous PDK1 was immunoprecipitated and immunoblotted with a PDK1 antibody. Whole cell lysates were probed with V5 and FLAG antibodies. **D**: Quantification of four independent experiments as in C. Indicated are the mean and standard error of the mean and three asterisks indicate T-test p value<0.001. **E**: HEK293T cells were transfected as indicated and USP4 was immunoprecipitated using a MYC antibody. Immunoprecipitations were incubated *in vitro* with purified HA-tagged PDK1/Ub-PDK1 as a substrate and immunoblotted with anti-HA antibody. The expression of the USP4 constructs was verified with anti-MYC antibody.
doi:10.1371/journal.pone.0031003.g004

inhibitors (Complete, Roche) and *N*-Ethylmaleimide (Sigma). The lysate was sonicated for 2×6 seconds on ice, centrifuged at 14.000 rpm for 10 min at 4°C and pre-cleared with 10 µl Protein A/G agarose beads (Pierce #20421). Subsequent immunoprecipitation was performed with 20 µl Protein A/G agarose bead suspension and 1 µg antibody per 1 ml lysate or pre-coupled agarose bead suspension (anti-HA agarose, Sigma A2095; anti-V5, Sigma A7345).

For isolation of His-tagged proteins, cells were lysed under denaturing conditions (10 mM Tris-HCl (pH 8.0), 6 M Guanidine-HCl, 100 mM NaH_2PO_4 and 1 mM Beta-Mercaptoethanol) supplemented with *N*-Ethylmaleimide and sonicated. Lysates were

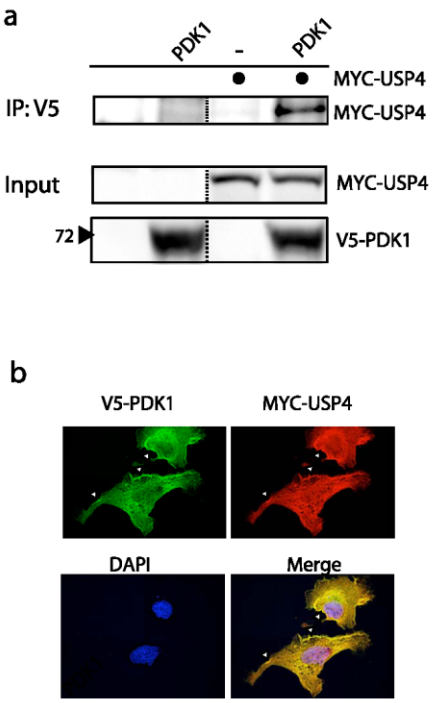

Figure 5. USP4 and PDK1 interact and co-localize at the plasma membrane. A: HEK293T cells were transfected as indicated and PDK1 was immunoprecipitated with anti-V5 beads. Immunoprecipitations (IP) and input were probed with anti-V5 and MYC antibodies. **B:** Confocal images from transfected U2OS cells stained with V5-PDK1 (green) and MYC-USP4 (red) antibodies. DNA was visualized by DAPI.
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incubated for 4 hours with nickel beads (Sigma H0537) and washed with imidazole buffer (10 mM imidazole, 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40 and 0.1% SDS). Beads were resuspended in sample buffer and boiled for 3 min at 95°C.

For immunoblotting, proteins were resolved by 4–12% Bis-Tris polyacrylamide gels (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in 2% i-Block (Applied Biosystem) or 4% BSA for 20 min and probed with the appropriate antibody overnight at 4°C. Detection of bound antibodies was performed by incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies at room temperature for 1 h followed by ECL according to the manufacturer's protocol (ECL-PLUS, GE Health Care).

Antibodies

Anti-PDK1 (sc-17765) and anti-GFP (sc-8334) were purchased from Santa Cruz. Anti-FLAG (F7425), anti-MYC 9E10 (M4439), anti-V5 V5-10 (V8012), anti-HA HA-7 (H9658) and anti-Actin (A2066) were obtained from Sigma. Anti-phospho-specific S241 PDK1 (#3061) and anti-HSP90 (SPS-771) were purchased from Cell Signaling and Stressgen, respectively. Anti-H1.2 (ab17677) was obtained from Abcam.

Plasmids and cell transfection

The pcDNA6-PDK1-V5-His vector was generated by cloning a PCR fragment with EcoRI and XbaI restriction sites (Fwd-primer 5′GAATTCGCCAGGACCACCAGCCAG3′, Rev-primer 5′TCACTGCACAGCGGCGTCTCTAGA3′) into the pcDNA6 backbone. All other mutants were generated by site-directed mutagenesis using the Stratagene Dpn1 PCR protocol.

The His-tagged ubiquitin expression vector was created by cloning human ubiquitin B into pcDNA3 using the restriction sites BamHI and XbaI. The PDK1-ubiquitin C-terminal fusion protein was created by PCR amplifying human ubiquitin B and cloning it 3′ of PDK1 in pcDNA6-PDK1-V5-His vector using KpnI and EcoRI.

PDK1 kinase domain (KD) expression vector was created by PCR amplification and cloning into pcDNA6-V5-His using EcoRI and XbaI. The following primers were used: Fwd-primer 5′-GATCGAATTCCCACCATGGCCAGGACCACCAGC-3′, Rev-primer 5′-GATCTCTAGACGGGTGAGCTTCGGAGGCGTC-3′.

HA-tagged ubiquitin expression vector (pRK5-HA-ubiquitin wild type) and the 68 DUB expression vectors were obtained from Addgene. cDNAs encoding the full-length USP4-WT and -C311S proteins in the pcDNA4-MYC-His vectors were kindly provided by Dr. Kristina Lindsten and subcloned into pcDNA6-V5-His. The K-less PDK1 mutant was ordered from Mr.Gene (Regensburg, Germany). All generated constructs and mutants were verified by Sanger sequencing.

Cells were transfected using the $CaPO_4$ method or with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions and cells were harvested between 48 and 72 hours post-transfection.

Cell fractionation was performed using the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem #539791) according to manufacturer's protocol.

In vitro deubiquitination assay

MYC-tagged USP4 was immunoprecipitated with anti-MYC antibody and co-incubated with purified STREP-HA-PDK1/Ub-PDK1 overnight at 30°C in a final volume for 15 µl of deubiquitination buffer (50 mM Hepes (pH 7.6), 100 mM NaCl, 5 mM $MgCl_2$, 5% glycerol, 0.2% Triton X-100, 10 mM DTT and 2 mM ATP). The reaction mixtures were analyzed by immunoblotting with the anti-HA antibody.

Confocal microscopy

U2OS cells were plated on glass coverslips in 6-well dishes and transfected with 4 µg cDNA using Lipofectamine 2000. After 48 hours, cells were fixed with 4% paraformaldehyde at room temperature for 15 min, permeabilized with 0.1% Triton X-100, blocked with 3% bovine serum albumin-phosphate-buffered saline solution and incubated with anti-V5 (Invitrogen R960-25) or anti-MYC 9E10 (Sigma C3956). After extensive washing, cells were incubated with Alexa-488- (Invitrogen A11001) and Alexa-564-conjugated (Invitrogen A10040) secondary antibodies or DAPI for 1 h. Coverslips were mounted onto slides with ProLong Gold (Molecular Probes). Immunofluorescence-stained cells were visualized with a Leica DMI6000B confocal microscope and images were captured with LAS AF software, version 2.3.0.

Supporting Information

Figure S1 The majority of DUB cDNA library is expressed in HEK293T cells. Cells were co-transfected with V5-tagged PDK1 and a DUB cDNA library. Whole cell lysates were probed with FLAG and MYC antibodies to verify the expression of DUBs.
(PDF)

Figure S2 Only USP4 shows a reproducible reduction in Ub-PDK1. HEK293T cells were co-transfected with V5-tagged PDK1 and indicated DUB cDNA clones. PDK1 was immunoprecipitated with anti-V5 beads and the Ub-PDK1 was detected using a V5 antibody. The normalized Ub-PDK1:PDK1 ratio is indicated below each lane. Whole cell lysates were probed with MYC and FLAG antibodies to verify the expression of DUBs. Asterisks indicate unspecific cross-reacting bands.
(PDF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: IZU TL SMBN. Performed the experiments: IZU TL SMBN. Analyzed the data: IZU TL SMBN. Contributed reagents/materials/analysis tools: IZU TL SMBN. Wrote the paper: IZU SMBN.

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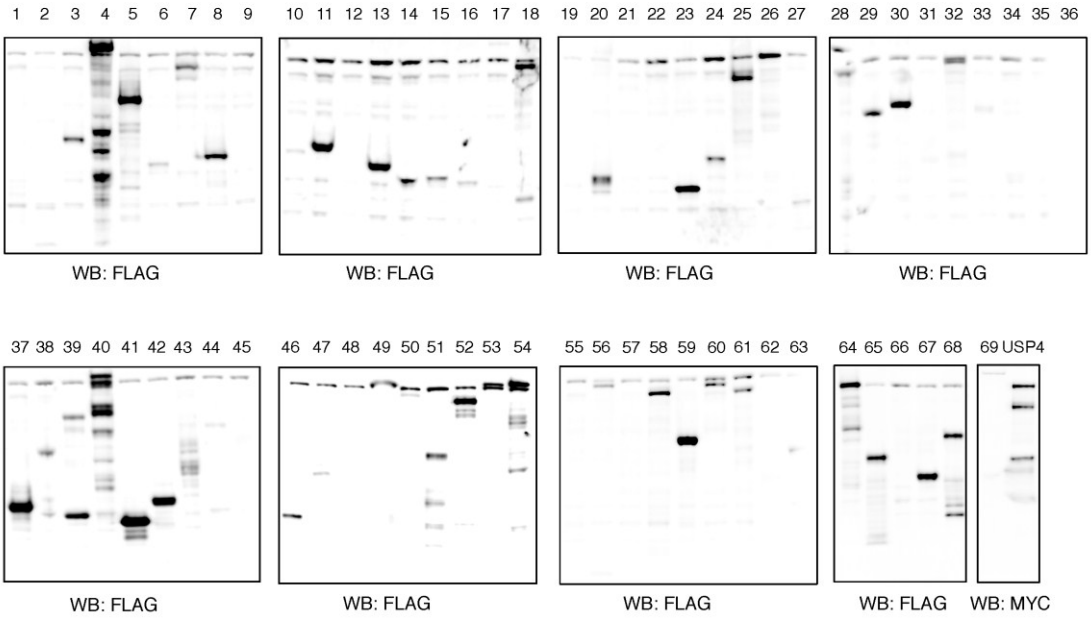
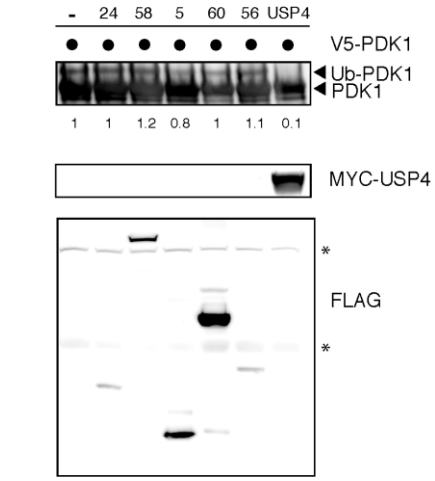
Supplementary Figures

S1



Supplementary Figures

S2



2.2.2.1 *Supporting information*

- Mass spectrometry analysis provides the possibility to reveal the ubiquitinated lysine residue(s) by detecting the m/z fingerprint of a peptide attached to a double glycine (GG) remnant. In order to avoid contaminations caused by other proteins in the whole cell extract and a noisy outcome in the fragment ion detection process, the ubiquitinated protein needs to be stringently purified. Here, to enrich the ubiquitinated PDK1, we took advantage of a Flp-In recombination system and established a cell line which upon doxycycline induction stably expressed StrepIII-HA-tagged PDK1. Unmodified and ubiquitin-conjugated PDK1 were purified by strep-tactin sepharose, cleaved with trypsin and analysed by mass spectrometry. The great majority of trypsin digested ubiquitinated PDK1 was detected (Figure 18). Yet, the target attachement site could not be identified.

MARTTSQLYDAVPIQSSVVLCSCPSPSMVRTQTESSTPPGIPGGSRQGPAMDGTAAEPRPGAGSLQHAQPP
PQPRKKRPEDFKFGKILGEGSFSTVVLARELATSREYAIKILEKRHIIKENKVPYVTRERDVMSRLDHPFF
VKLYFTFQDDEKLYFGLSYAKNGELLKYIRKIGSFDETCTRFYTAEIVSALEYLHGKGIIHRDLKPENILL
NEDMHIQITDFGTAKVLSPESKQARANSFVGTAQYVSPELLTEKSACKSSDLWALGCIIYQLVAGLPPFRA
GNEYLIFQKIIKLEYDFPEKFFPKARDLVEKLLVLDATKRLGCEEMEGYGPLKAHPFFESVTWENLHQQTP
PKLTAYLPAMSEDDEDCYGNYDNLLSQFGCMQVSSSSSSHSLSASDTGLPQRSGSNIEQYIHDLDSNSFEL
DLQFSEDEKRLLLEKQAGGNPWHQFVENNLILKMGPVDKRKGLFARRQLLLTEGPHLYYVDPVNKVLKGE
IPWSQELRPEAKNFKTFFVHTPNRTYYLMDPSGNAHKWCRKIQEVWRQRYQSHPDAAVQ

Figure 18. The majority of predicted tryptic peptides of PDK1 is detected by mass spectrometry

The sequence coverage (78%) is underlined and highlighted in bold. Lysine residues that are not detected are shown in red.

- The PI3K pathway is implicated in insulin signalling, triggering phosphorylation of PDK1 which in turn activates survival pathways by phosphorylating its downstream substrates. To investigate the influence of insulin signalling on PDK1 mono-ubiquitination we stimulated cells with insulin and immunoprecipitated endogenous PDK1. Activation of PDK1 pathway was determined by phosphorylation of its well-known substrate AKT at Thr308. However, no clear difference in ubiquitin-conjugated PDK1 (Ub-PDK1) was obtained between stimulated and starved cells (Figure 19), indicating that insulin signalling does not regulate PDK1 ubiquitination at least under conditions we tested.

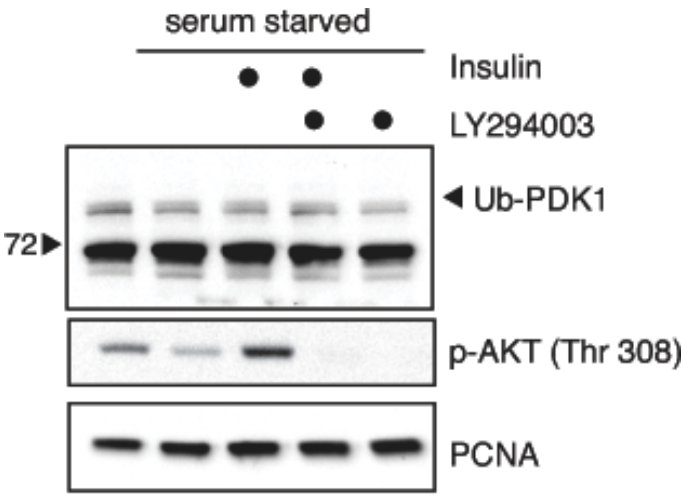


Figure 19. PDK1 mono-ubiquitination does not alter upon insulin stimulation

HEK293T cells starved overnight were either left serum-starved or stimulated with insulin for 8 hours. A PI3K inhibitor, LY294003, served as a control. Immunoprecipitated endogenous PDK1 was detected with anti-PDK1 antibody. Signalling activation was determined by an antibody specific for phosphorylated AKT at the Thr308 site. PCNA (proliferating cell nuclear antigen) was used as a loading control.

- To further characterize whether PDK1 mono-ubiquitination depends on its kinase activity, we analysed a PDK1 activation loop point mutant at serine 241 as this site is known to phoshorylate itself and as a consequence, to be crucial for PDK1 activity (Casamayor et al, 1999). Interestingly, PDK1^{S241A} could no longer be ubiquitinated (Figure 20) and in agreement with previous studies was not phosphorylated (Casamayor et al, 1999). This observation indicated a crosstalk between PDK1 T-loop phosphorylation and mono-ubiquitination and further suggested that PDK1 ubiquitination does not require kinase activity.

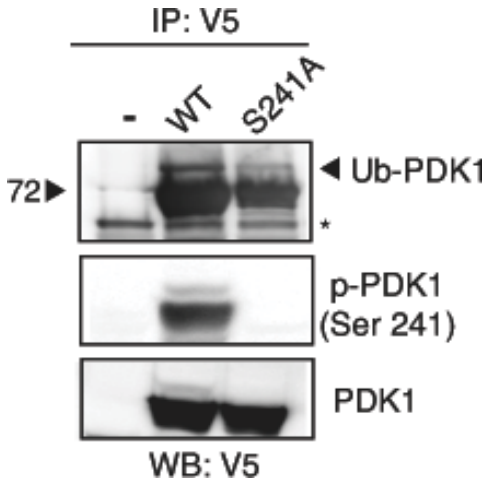


Figure 20. T-loop mutation of PDK1 at Ser241 inhibits mono-ubiquitination

HEK293T cells were transfected as indicated. The activation loop mutant S241A of PDK1 was immunoprecipitated with anti-V5 beads and detected with anti-V5 antibody. A phospho-specific antibody was used to confirm PDK1 phosphorylation at the Ser241 residue. The wild type construct (WT, V5-PDK1) served as a control. Asterisk indicates unspecific cross-reacting band.

3 Discussion

In this study, we focused on different aspects of how to identify new and better stratified interventions in cancer treatment.

In the first part of this thesis, we generated a screening platform to systematically link tumour genotype with drug response. Our combinatorial gene-small compound screen dataset analysis unveiled various synthetic lethal interactions that may provide novel and better angles for personalized treatment with few side effects. We also disclosed a new mechanism of resistance to PI3K inhibitors upon NOTCH activation and c-MYC induction. As these inhibitors are currently in clinical trials, our data have direct clinical impacts on breast cancer treatment (Muellner et al, 2011).

It is worthy to note that a large cohort of breast cancer patients express high levels of oncogenic PI3K pathway activation which correlate with poor prognosis (Liu et al, 2009; Workman et al, 2010). In particular, PDK1, a critical component of this signalling cascade, is found overexpressed in many breast tumours. In addition, its prominent substrate AKT favours tumourigenesis when aberrantly activated by excessive growth factors or oncogenic mutations. Henceforth, to search for a new therapeutic window targeting the PI3K-PDK1-AKT pathway, in the second part of this thesis we zoomed into PI3K signalling cascade and gained highly promising insight into the PDK1 regulation. In fact, this study for the first time described mono-ubiquitination as a novel mechanism to regulate PDK1 (Uras et al, 2012). We also unveiled USP4 as a novel deubiquitinase targeting PDK1 for deubiquitination *in vivo* and *in vitro* in a deubiquitinase activity dependent manner (Uras et al, 2012). Given that interfering with the ubiquitination machinery is considered as an exciting targeting strategy for cancer therapy (Cohen & Tcherpakov, 2010), our data provides potential novel angles for therapeutic applications (Uras et al, 2012).

3.1 Luminex-based combinatorial genetic and chemical screening in breast cancer cells

We developed a screening strategy to systematically search for selective toxicity towards any desired tumour genotype (synthetic lethal interactions) and resistance mechanisms of therapeutic agents in cancer cells. The ability to link tumour genotype with drug response, thus to individualize the treatment of cancer, is considered as a prime tool to rationally select patients for therapies and impacts on society and economy at several levels (Bernards, 2010; Fojo & Grady, 2009; Majewski & Bernards, 2011; Muellner et al, 2011). Indeed, considering rising cost of therapeutic agents and care of cancer patients -the life of all cancer patients who die in the United States annually could be extended by one year for $ 440 billion-, this ability is not only a clinical necessity but also an economic requirement to keep the highly increasing cost of cancer treatment in check by reducing the number of patients experiencing no therapeutic benefits (Bernards, 2010; Fojo & Grady, 2009; Majewski & Bernards, 2011; Muellner et al, 2011). Hence, understanding the complex interplay between the molecular make-up of a patient‘s tumour and the therapeutic response remains a critical goal of cancer research. Unveiling such interactions can also yield unexpected cancer vulnerabilities which may assist to define drug sensitive patient subpopulations and guide more directed clinical trials or aid in drug repositioning. Our multiplexed assay allowed us to interrogate thousands of combinations of genetic and chemical perturbations with the potential to uncover clinically relevant interactions that may indeed assist the development for new patient-tailored medicine. The method proves to be fast, cost effective and highly flexible and can be used with cDNA overexpression, RNAi or any cellular perturbation of interest (Muellner et al, 2011). This specific assay is applicable to all cells transducible with lentiviral vectors and can also be used to study biological questions that are not strictly related to synthetic lethality (Muellner et al, 2011).

To model the aberrant gene regulation found in breast cancer patients with a great prudence, we decided to make use of an isogenic cell line approach rather than using heterogeneous conventional cell lines that may mask the effect of individual genetic changes on cellular viability -that are synthetic lethal or resistant pairs- due to their multitudes of genetic changes (Nijman, 2011). We have carefully created a panel of engineered isogenic MCF10A cell lines, each differing from the parent only in one single genetic change. We subjected the parental MCF10A cells, an immortal non-tumourigenic breast epithelial line, to several criteria to confirm its suitability and

thus power for genetic screening. MCF10 cells exhibit untransformed and non-invasive properties and highly contact-inhibited growth and express breast-specific antigens and wild type p53 (Neve et al, 2006; Soule et al, 1990). Of note, genome-wide human SNP array analysis unveiled several abnormalities in MCF10A cells such as trisomies, amplified and deleted chromosomal fragments or entire arms.

Nevertheless, compared to studies about genomic aberrations underlying breast cancer, abnormalities monitored in MCF10A cells showed very few matches in loci frequently affected in patients (Bergamaschi et al, 2006; Hicks et al, 2006). Hence, the MCF10A cells qualified as relatively normal. A further advantage of untransformed cells is that the ectopic expression of oncogenes or the inhibition of tumour suppressor genes by RNAi likely has a physiological downstream consequence. Therefore, we investigated phenotypic transition upon altered gene expression. Strikingly, isogenic MCF10A shRNA cell lines monitored did not induce any phenotypic changes during a 4 weeks period compared to wild type which may be potentially explained by the not sufficient enough knock-down of target gene expression by RNAi. However, encouragingly, upon long-term incubation for up to 6 weeks post-infection, a number of MCF10A isogenic cell lines stably transduced with oncogenes including $HRAS^{V12}$ and myristoylated AKT1 exhibited a marked morphological change indicative for oncogenic cellular transformation (data not shown). These observed oncogene-induced transitions were very well in agreement with previously published reports (Blanco-Aparicio et al, 2010; Braig & Schmitt, 2006; Jacobs et al, 1999; Manning & Cantley, 2007; Mason et al, 2004; Mende et al, 2001; Wang et al, 2009). Along this line of reasoning, we concluded that the genetic background of MCF10A cells allow us to stimulate maximal rewiring of signalling pathways and alteration of cellular state upon introduction of breast cancer-relevant driver mutations, thus the engineered cell line panel is suitable for simulating the heterogenous mutational landscape across breast cancer patients (Muellner et al, 2011).

We subjected this “synthetic” cancer model to different screens. The MCF10A panel was used to search for synthetic lethalities in dependency on human protein kinases using a siRNA library. Such a screening effort is comparable to synthetic lethality screens performed in yeast to uncover genes causing genotype-specific cell death (Bender & Pringle, 1991; Torrance et al, 2001). In parallel, we wished to investigate potential conservation of synthetic lethal interactions across different cancer types. Such a study is conceivable as a) microarray analysis of the NCI60 cell lines

revealed that the majority of cancer genes are expressed in most tissues, b) a panel of human cancer cell lines display conserved vulnerabilities to some small molecules (Barretina et al, 2012; Garnett et al, 2012), c) few synthetic lethal interactions found in yeast are evolutionary conserved in the nematode *C. elegans* (Byrne et al, 2007; Nijman, 2011; Tarailo et al, 2007) and d) limited conservation of genetic interactions are observed between human cell lines and yeast or *C. elegans* with the potential to identify novel targets for anti-cancer therapeutic development (Conde-Pueyo et al, 2009; McLellan et al, 2009; McManus et al, 2009; Nijman, 2011; van Pel et al, 2013; Yu et al, 2008). With this goal, an isogenic cell line panel based on HeLa cells, a cervix carcinoma cell line, was engineered upon introduction of individual shRNAs targeting common tumour suppressors and subsequently screened against the same kinase siRNA library as used in the MCF10A gene-gene synthetic lethality screen (data not shown). A third approach to unveil synthetic lethalities that may help to explain the variability in therapy response observed among patients was presented by screening the MCF10A isogenic cells against a clinically relevant drug library of mainly kinase inhibitors and different tool compounds.

Our top scoring findings obtained from both MCF10A- and HeLa-based kinase siRNA genetic screens, however, could not be recapitulated in multiple independent experimental settings. The failure to demonstrate those interactions may be explained by technical limitations such as suboptimal experimental conditions of validation and follow-up studies. In some cases, the difference in viability between mutant and wild type cells was not very pronounced thus could probably not be qualified to be therapeutically exploited. It is also not clear to which extent false-positive hits can contaminate screening results. In addition, false-negative hits may be due to insufficient knock-downs by RNAi. Even though high-throughput cell-based RNAi screens in *Drosophila* and human cells have clearly unfolded valuable discoveries, the dataset interpretation still remains highly challenging (Kaelin, 2012; Mohr et al, 2010). Many potential drug targets discovered in academic laboratories by RNAi technology fail in clinical trials (Begley & Ellis, 2012; Kaelin, 2012; Prinz et al, 2011). It is not uncommon that assays designed with the same goal, for instance, to kill cells carrying a particular genetic modification but using different libraries and different ranking algorithms yield very different discoveries (Barbie et al, 2009; Barrows et al, 2010; Goff, 2008; Kaelin, 2012; Luo et al, 2009a; Scholl et al, 2009). The validity of RNAi outcome may be improved when more focused screens employing less than 100 genes are performed. This would surely enable a deeper interrogation of primary top scoring hits in lower-throughput secondary screens

(Kaelin, 2012). The analysis and verification of RNAi results and validation of their biological relevance can also be enhanced by a) better methods for RNAi reagent design and delivery and for large-scale experimental verification of mRNA knockdown, b) improved genome-wide RNAi libraries with increased numbers of siRNAs per gene and elimination of the constructs frequently monitored to cause false discoveries across multiple independent screens, c) scoring/ranking algorithms that incorporate knock-down efficiencies and orthogonal datasets and d) computational methods to analyse perturbation phenotypes that do not average out cell-to-cell variability but account and incorporate cell population context and thus single cell microenvironment (Kaelin, 2012; Mohr et al, 2010; Snijder et al, 2012).

The combined synthetic lethal and drug resistance screen in MCF10A isogenic cell line panel yielded numerous pharmaco-genetic interactions with the potential to guide the treatment choice and predict outcome. For instance, TWIST cells showed hypersensitivity upon JAK2 inhibition, which may be explained by the requirement of both TWIST and JAK2/STAT3 for breast cancer stem cells (Marotta et al, 2011; Vesuna et al, 2009). Furthermore, we observed that abnormal activation of RAS or PI3K uncoupled EGFR signalling from proliferation and as a consequence resulted in resistance to EGFR tyrosine kinase inhibitor. This observation has clinical relevance as the EGFR inhibitor Gefinitib, even though not approved by FDA yet, seems to be effective to treat metastatic breast tumours when combined with other chemotherapeutics (Bernsdorf et al, 2011). Moreover, we monitored a synthetic lethal interaction between NOTCH pathway and Aurora kinase inhibitors (Muellner et al, 2011). This was in agreement with previous studies (Yang et al, 2010a) and hence further illustrated the power of our screening platform. In addition, the fact that several components of a single signalling pathway clustered with two independent compounds targeting the same demonstrated how successfully high-throughput chemical-genetic screens can advance our understanding of mechanisms behind drug actions and cancer gene functions (Muellner et al, 2011).

Of particular note, a potential handicap of engineered cells is that they are artificially generated. Henceforth, they may not rigorously experience the full tumour evolution process and thus the genetic background and cellular state may not truly mimic a “normal” cancer cell (Nijman, 2011). This notion may elucidate why some previously defined oncogene addiction hits were not recapitulated in our assay. Furthermore, knock-down insufficiencies or other technical limitations may have caused false discoveries. This may explain, for instance, why the previously described resistance

mechanism to PI3K inhibitors due to PTEN deficiency (Brachmann et al, 2009) was not recapitulated in our setting. Nevertheless, the fact that multiple cell lines shared mechanisms of resistance and synthetic lethal interactions justifies our assay and highlights the power of isogenic models (Muellner et al, 2011).

We were among the first academic laboratories who successfully unveiled a new resistance to PI3K inhibition in breast cancer cell lines and proposed that acquired resistance is driven by activated NOTCH signalling and c-MYC induction (Muellner et al, 2011). It is worthy to note that NOTCH activation is found in a large fraction of aggressive breast tumours and associated with poor overall survival and that MYC amplification frequently favours tumourigenesis (Al-Hussaini et al, 2011; Deming et al, 2000; Muellner et al, 2011; Reedijk, 2012; Reedijk et al, 2005). Because of the high frequency of aberrant oncogenic activation of PI3K-mTOR signalling in a variety of malignancies including breast cancer, development of effective pharmacological inhibitors targeting this pathway has received much attention (Keniry & Parsons, 2008; Liu et al, 2009; Moasser, 2007; Muellner et al, 2011; Sauter et al, 1996; Tornillo & Terracciano, 2006; Yuan & Cantley, 2008). In fact, several PI3K targeting small molecules including BEZ-235 have entered clinical trials (Kong & Yamori, 2009; Muellner et al, 2011; Serra et al, 2008). Because acquired resistance to single agents within tumour cells presents an emerging clinical hurdle, the capability to predict drug resistance holds great value not only at clinical but also economic level (Muellner et al, 2011). Potential mechanisms of drug resistance in human tumours to PI3K inhibition, however, have not yet been documented at the time of this study. We could demonstrate that transcriptionally activated c-MYC uncouples cellular proliferation from mTOR regulation of translation and subsequently mediates resistance to PI3K/mTOR inhibitors (Muellner et al, 2011). How c-MYC drives protein translation by inducing eukaryotic initiation factor 4F (eIF4) family members is a well-documented mechanism and is linked with c-MYC-induced carcinogenesis (Jones et al, 1996; Lin et al, 2009; Muellner et al, 2011; Ruggero & Pandolfi, 2003; Schmidt, 2004). The mechanism of how activated NOTCH1 could indeed mediate resistance to PI3K targeting drugs presents an exciting model and requires further validations (Muellner et al, 2011). Importantly, a parallel study has reported that genomic amplification of either *MYC* or its downstream effector *eIF4E* proved sufficient to provide resistance to PI3K inhibitors and was indicative for upregulated cap-dependent translation in resistant breast cancer cells, further corroborating our results (Ilic et al, 2011). Moreover, we provided evidence for the conservation of the observed resistance mechanism among a variety of tumour types (Muellner et al,

2011). In line with this, a recent study reported NOTCH-MYC induced resistance to PI3K/mTOR inhibition in T-cell acute lymphoblastic leukemia, further corroborating and validating our findings (Shepherd et al, 2013).

In conclusion, we present a strategy to mimic abnormal regulation of cancer underlying driver genes and describe a screening platform, which presents a valuable tool to tailor patient responsiveness and to identify new approaches to fighting cancer. We propose activation of NOTCH and MYC signalling as potential mechanisms of resistance to PI3K inhibitors with direct clinical impact and hence demonstrate the power of large-scale measurements of genetic interactions in human cells with the potential to offer deeper insight into different facets of chemical biology (Muellner et al, 2011).

3.2 USP4 regulates PDK1 mono-ubiquitination

We show for the first time that PDK1 is modified by a single ubiquitin to a variable extent in a broad range of human cell lines, suggesting that it may play an important role in PDK1 regulation (Uras et al, 2012). Because of the crucial role PDK1 has in signalling to dictate many vital cellular processes, we investigated whether PDK1 ubiquitination can be modulated by growth factor signalling. However, we did not obtain evidence for that. Total ubiquitinated PDK1 did not alter in response to serum starvation or stimulation with insulin, EGF or glucose (data not shown). Of note, PDK1 pathway activation was determined by phosphorylation of its well-known substrate AKT at Thr308. Nonetheless, mono-ubiquitination might steer PDK1 activity towards downstream targets other than AKT, which remains to be tested.

PDK1 is also known to shuttle between nucleus and cytoplasm in a CRM1-dependent and growth factor-regulated manner even though the functional significance of this has not been fully understood (Lim et al, 2003; Scheid et al, 2005; Uras et al, 2012). In addition, mono-ubiquitination has been documented to orchestrate subcellular localization (Li et al, 2003). This line of reasoning prompted us to investigate a potential role of mono-ubiquitination in PDK1 trafficking. However, no compartment-specific response could be observed as ubiquitinated PDK1 was equally distributed over the nuclear, cytoplasmic and membrane fractions upon insulin or EGF stimulation (data not shown). Perhaps alternative cellular fractionation

assays with higher resolution such as endosomal isolation may unravel a differential distribution.

Nevertheless, it is just logical to assume that only specific conditions or certain stimuli have the power to modulate PDK1 ubiquitination (Uras et al, 2012). In fact, there is enough evidence that mono-ubiquitination is crucial for protein functionality. In response to DNA damage, for instance, the Fanconi Anemia protein FANCD2 becomes mono-ubiquitinated and subsequently shuttles to the chromatin, while ubiquitin-conjugated proliferating cell nuclear antigen (PCNA) attracts a specific DNA polymerase (Huang & D'Andrea, 2006; Kannouche et al, 2004; Uras et al, 2012). Moreover, mono-ubiquitination of tyrosine kinase receptors regulates receptor endocytosis and p53 mono-ubiquitination stimulates its accumulation in the cytoplasm (Li et al, 2003; Marmor & Yarden, 2004; Uras et al, 2012). Hence, future research will certainly define a key regulatory function of PDK1 mono-ubiquitination. This function will have particular significance as to date it is not comprehensively characterized how PDK1 dictates the activation of its many targets (Mora et al, 2004; Uras et al, 2012). Indeed, PDK1 may induce subtle changes in subcellular localization or modulate binding to adapter molecules and downstream substrates in a ubiquitination-dependent manner (Uras et al, 2012). Perhaps co-immunoprecipitations of known substrates with PDK1 and subsequent detection of PDK1 mono-ubiquitination state in these protein complexes may provide more insight on whether this post-translational modification drives PDK1 activity towards different substrates.

However, the lack of a PDK1 point mutant that is no longer mono-ubiquitinated hindered us to study these hypotheses. Re-introduction of individual lysine residues in the lysine-less PDK1 mutant did not re-establish ubiquitination (Uras et al, 2012). A plausible explanation for this may be that interaction with the responsible ubiquitin E3 ligase is hampered when all lysines in the kinase domain are mutated. The reason why the reverse experiment in which conserved lysine residues were mutated to arginines did not yield the target site on PDK1 may be due to redundancy between target lysines as has been documented for AKT, p53 and cyclin B (Chan et al, 2006; King et al, 1996; Uras et al, 2012; Yang et al, 2009). This surely makes both the genetic and biochemical assays to define the ubiquitin-conjugation site(s) highly laborious (Uras et al, 2012). Moreover, mass spectrometry experiments with purified and trypsin digested ubiquitinated PDK1 did not unveil the target residue, even though the great majority of predicted tryptic peptides was detected (Uras et al,

2012). Several factors may contribute to the lack of success. Indeed, ubiquitin-modified proteins can also simultaneously be attached to other ubiquitin-like modifiers, which can leave GG remnants just like ubiquitin after tryptic digestion (Xirodimas et al, 2004). Because detection depends on mass-shift caused by these remnants, loss of modifier identity represents a major limitation. Furthermore, because only a small proportion of total PDK1 is ubiquitin-conjugated - its abundance is weaker by a factor of approximately 50-, little amounts of possible contaminants may easily generate noise and influence the detection efficiency of mass spectrometry. Nonetheless, we believe that future mass spectrometry analyses using alternative proteases will decipher the ubiquitin attachement site(s) on PDK1 and facilitate a more systematic functional analysis (Uras et al, 2012).

We noted that ubiquitination occurs in PDK1 kinase domain (Uras et al, 2012). Yet this modification does not depend on PDK1 kinase activity as a catalytically inactive PDK1 point mutant was still modified by a single ubiquitin moiety (Uras et al, 2012). Thus, kinase activity does not control ubiquitin-conjugation on PDK1, at least under these conditions (Uras et al, 2012). In addition, a PDK1 activation loop point mutant at the residue serine 241 which serves as an auto-phosphorylation site essential for PDK1 activity, inhibited mono-ubiquitination, indicating a potential crosstalk between the two post-translational modifications, phosphorylation and ubiquitination, of PDK1. This observation also suggested that $PDK1^{S241A}$ might display a greater affinity to potential deubiquitinases compared to wild type.

Given that mono-ubiquitination does not result in protein degradation and can be in fact reverted by deubiquitinases, we screened the majority of human deubiquitinases for their ability to interfere with PDK1 ubiquitination and discovered USP4 as the only DUB targeting PDK1 *in vivo* and *in vitro* in a deubiquitinase activity dependent manner (Uras et al, 2012). We qualified USP4 as a prime regulator of ubiquitinated PDK1 by direct deubiquitination. We further substantiated PDK1 as a specific USP4 substrate by proving a robust physical interaction between the two proteins (Uras et al, 2012). Moreover, this interaction was irrespective of a PDK1 point mutation at serine 241 (data not shown), indicating that the inactive PDK1 T-loop mutant may exhibit a higher affinity to potential ubiquitin conjugating E2 enzymes and/or ubiquitin E3 ligases that modulate PDK1 ubiquitination. The E3 ligase specific for PDK1 may theoretically be uncovered by affinity purification of PDK1 with its interactors from cell lysate under non-denaturing conditions and subsequent analysis by shotgun mass spectrometry (Domon & Aebersold, 2006). In addition, confocal microscopy analysis

revealed that USP4 and PDK1 co-localize intensely at the plasma membrane upon overexpression of both proteins, further supporting a direct deubiquitination of PDK1 by USP4 (Uras et al, 2012). Moreover, inhibition of PDK1 ubiquitination by ectopically expressed USP4 did not induce any changes in response to stimulation with insulin, EGF or glucose (data not shown).

USP4 has a critical role in a variety of processes including protein quality control in the endoplasmatic reticulum and p53, TGF-β, NF-κB and Wnt signalling (Aggarwal & Massague, 2012; Fan et al, 2011; Milojevic et al, 2006; Uras et al, 2012; Xiao et al, 2012; Zhang et al, 2012; Zhang et al, 2011; Zhao et al, 2009). Of interest, USP4 has been reported to inhibit the kinase TAK1 that becomes ubiquitinated by TRAF6, which is an important AKT regulator and is also targeted by USP4 for deubiquitination (Liang et al, 2013; Uras et al, 2012; Yang et al, 2009; Yang et al, 2010b). A direct association between USP4 and AKT has also recently been documented (Zhang et al, 2012). In particular, AKT-driven phosphorylation of USP4 stimulates its subcellular re-localization from the nucleus to the membrane, increasing its stability and thus deubiquitinase activity (Zhang et al, 2012). Together, USP4 may impact on the PI3K-PDK1-AKT signalling at multiple levels (Uras et al, 2012). However, USP4 depletion did not alter PDK1 ubiquitination state (data not shown). This may be simply due to technical limitations that partial inhibition of USP4 by RNAi is not sufficient enough to detect changes in ubiquitinated PDK1. Other possible explanations may be provided by low basal activity of endogenous USP4 or presence of other functionally redundant DUBs (Uras et al, 2012).

Of great value, USP4 has been implicated in several pro-tumourigenic functions. Indeeed, USP4 is highly expressed in a variety of human tumours including breast cancer, qualifying USP4 as a potential oncogene (Zhang et al, 2011). USP4 is also considered as an antagonist of lung cancer cell migration and a potential therapeutic target for head and neck squamous cell carcinoma (HNSCC) (Fan et al, 2011; Hou et al, 2013; Xiao et al, 2012; Zhou et al, 2012). Of note, USP4 depletion can cause inhibition of AKT-mediated breast cancer cell migration, proposing to develop USP4-specific inhibitors, which would hinder breast cancer cells to invade and metastasize (Zhang et al, 2012).

In conclusion, we report mono-ubiquitination as a novel post-translational modification of PDK1, which can be directly reverted by the ubiquitin-specific protease 4. The newly identified role of USP4 as a highly specific modulator of PDK1

just increases the complexity of the PDK1 signalling network and provides potential exciting angles for drug discovery (Uras et al, 2012).

4 Materials and Methods

The section “Materials and Methods” is written according to the publications “A chemical-genetic screen reveals a mechanism of resistance to PI3K inhibitors in cancer” (Nat Chem Biol. 2011 Sep 25;7(11):787-93. doi: 10.1038/nchembio.695) (Muellner et al, 2011) and “Ubiquitin-specific protease 4 inhibits mono-ubiquitination of the master growth factor signaling kinase PDK1” (PLOS ONE. 2012;7(2):e31003. doi: 10.1371/journal.pone.0031003) (Uras et al, 2012).

4.1 Materials

Special materials used in this thesis are listed in detail below. Primers were purchased from Sigma-Aldrich.

Table 14: Buffer solutions

Buffer	Component	Concentration	pH
Lysis buffer for genomic DNA isolation	SDS	1 %	
	EDTA	10mM	
	Tris-HCl	10mM	8
	NaCl	10mM	
Tris-EDTA (TE buffer)	Tris-HCl	10mM	8
	EDTA	1mM	
1.5 x TMAC	Tetramethylammonium chloride	4.5 M	
	N-lauryl sarcosine	0.15 %	
	Tris-HCl	75mM	8
	EDTA	6mM	
1x TMAC	Tetramethylammonium chloride	3M	
	N-lauryl sarcosine	0.1 %	
	Tris-HCl	50mM	8
	EDTA	4mM	
RIPA	Tris-HCl	25mM	7.6
	NaCl	150mM	
	Tergitol	1%	
	Triton X-100	0.5%	
	SDS	0.1%	
ELB	NP-40	0.1%	
	HEPES	50mM	
	NaCl	250mM	
	EDTA	5mM	
Lysis buffer to isolate His-tagged proteins	Tris-HCl	10mM	8
	Guanidine-HCl	6M	
	NaH_2PO_4	100mM	
	Beta-Mercaptoethanol	1mM	
Imidazole buffer	Imidazole	10mM	
	Tris-HCl	25mM	8

	NaCl	150mM	
	NP-40	1%	
	SDS	0.1%	
Deubiquitination buffer	HEPES	50mM	7.6
	NaCl	100mM	
	$MgCl_2$	5mM	
	Glycerol	5%	
	Triton X-100	0.2%	
	DTT	10mM	
	ATP	2mM	
4 x SDS sample buffer	Tris-HCl	320mM	6.8
	Glycerol	40%	
	Bromphenol blue	16µg/ml	
	SDS	8%	
	Beta-Mercaptoethanol	20%	
HEPES buffered saline (HBS)	NaCl	1.4M	7.1
	HEPES	250mM	
	NaH_2PO_4	7.5M	

Table 15: Special reagents

Reagent	Short description	Company	Article number
D-(+)-Biotin 98%	Protein elution	Alfa Aesar	A14207-03
RNase A	Ribonuclease, RNA degradation	Applichem	
I-Block™	Protein-based blocking reagent	Applied Biosystems	T2015
PI(3,4,5)P3 PIP Beads™	Agarose of bound $PI(3,4,5)P_3$, for pull-down assays to detect and characterize phosphoinositide-binding proteins	Echelon Biosciences	P-B345a
dNTPs	dATP, dGTP, dCTP, dTTP mix	Fermentas	
Polyvinylidene difluoride (PVDF) membrane	Protein transfer, blotting membrane	GE Healthcare	RPN2020F
Strep-Tactin® Sepharose® 50% suspension	Gravity flow purification of recombinant *Strep*-tag® proteins	IBA	2-1201-010
NuPAGE® Novex® 4-12% Bis-Tris Gels	High-performance polyacrylamide gel	Invitrogen	

	electrophoresis system, stimulates denaturing conditions without using SDS detergent		
NuPAGE® MOPS SDS Running Buffer (20X) (Novex®)	Run proteins on NuPAGE® Novex® Bis-Tris gels only	Invitrogen	NP0001
SAPE	Biotin detection	Invitrogen	S-866
xMAP Technology microspheres	Colour coded, multiplexing of bioassays	Luminex	
ProLong ® Gold Antifade Reagent	Mounting medium, enhanced resistance to photobleaching	Molecular Probes	P36930
Restriction enzymes	DNA digestion	New England Biolabs	
Protein A/G agarose beads	Purification and detection of mouse monoclonal antibodies from IgG subclasses	Pierce	#20421
cOmplete protease inhibitor cocktail tablets	Protection of proteins against a broad range of proteases	Roche Applied Science	11697498001
Anti-V5 Agarose Affinity Gel antibody produced in mouse	Affinity purification and immunoprecipitation of V5-tagged fusion proteins	Sigma-Aldrich	A7345
Monoclonal Anti-HA−Agarose antibody produced in mouse clone HA-7	Immunoprecipitation and immunoaffinity purification of HA-tagged fusion proteins	Sigma-Aldrich	A2095
ANTI-FLAG® M2 Affinity Gel	Purification or immunoprecipitation of FLAG fusion proteins	Sigma-Aldrich	A2220
HIS-Select® HF Nickel Affinity Gel	Purification of histidine tagged proteins in native and denaturing conditions	Sigma-Aldrich	H0537
Sephadex® G-50	Gel filtration medium	Sigma-Aldrich	G5050
N-Ethylmaleimide	Covalent modification of cysteine residues in proteins	Sigma-Aldrich	E3876
DAPI	Fluorescent stain, binds to A-T rich regions of DNA, used in fluorescence microscopy	Sigma-Aldrich	D9542
Proteinase K	Serine protease, protein degradation	Sigma-Aldrich	

Table 16: Kits

Kit	Component	Short description	Company	Catalogue number
Agencourt Genfind v2	Lysis buffer Wash I buffer Wash II buffer Binding buffer Proteinase K Proteinase K buffer	Genomic DNA isolation	Beckman Coulter	A41497
MinElute Gel Extraction	MinElute spin columns Buffer QG Buffer PE Buffer EB Collection tubes Loading dye	Gel extraction of up to 5µg DNA fragments (70bp to 4kb) in low elution volumes	Qiagen	28604
Plasmid Kit	QIAGEN-tip Buffer P1 Buffer P2 Buffer P3 Buffer QBT Buffer QF RNase A LyseBlue	Purification of plasmid DNA	Qiagen	12125 12145 12165
RNeasy MinElute Cleanup	RNeasy MinElute spin columns Collection tubes Buffer RLT Buffer RPE RNase-free water	RNA cleanup and concentration with small elution volumes	Qiagen	74204
TURBO DNA-*free*	TURBO DNase DNase inactivation reagent 10 x reaction buffer Nuclease-free water	Genomic DNA removal prior to RT-PCR	Ambion	AM1907M
BigDye® Terminator v3.1 Cycle Sequencing Kit	5 x BigDye buffer BigDye	DNA sequencing	Applied Biosystems	4337454
RevertAid First Strand cDNA Synthesis	RevertAid reverse transcriptase RiboLock RNase	Reverse transcription	Fermentas	K1621

	inhibitor 5 x reaction buffer dNTP mix Oligo $(dT)_{18}$ primer Random hexamer primer Control *GAPDH* RNA 10 µM forward GAPDH primer Nuclease-free water			
iTaq SYBR Green Supermix With ROX	dNTPs iTaq DNA polymerase 6mM Mg^{2+} SYBR Green I ROX reference dye	Real-time PCR mix for use on ROX-dependent optical thermal cyclers	Bio-Rad	172-5851
siLentFect™ Lipid	siLentFect™ lipid reagent for RNAi	RNAi-specific lipid transfection	Bio-Rad	170-3362
Lipofectamine® 2000	Lipofectamine® 2000 transfection reagent	DNA or RNAi transfection	Invitrogen	11668-019
GoTaq® G2 DNA Polymerase	DNA polymerase 5 x Green GoTaq® reaction buffer 5 x colorless GoTaq® reaction buffer	PCR	Promega	M7841
CellTiter-Glo®	CellTiter-Glo® reagent	Luminescent cell viability assay	Promega	G7572
ECL Plus Western Blotting Reagents	ECL plus solution A ECL plus solution B	Chemiluminescent detection	GE Healthcare	RPN2133
Pierce ECL Western Blotting Substate	Luminol/enhancer Stable peroxide buffer	Detection of HRP enzyme activity	Thermo Scientific	32106
QuikChange® Site-Directed Mutagenesis	*PfuTurbo*® DNA polymerase (2.5 U/µl) 10 x reaction buffer *Dpn* I restriction enzyme (10 U/µl) Oligonucleotide	In vitro site-directed mutagenesis	Stratagene	#200518

	control primer #1 [34-mer (100 ng/µl)] 5´ CCA TGA TTA CGC CAA GCG CGC AAT TAA CCC TCA C 3´ Oligonucleotide control primer #2 [34-mer (100 ng/µl)] 5´ GTG AGG GTT AAT TGC GCG CTT GGC GTA ATC ATG G 3´ pWhitescript™ 4.5-kb control plasmid (5ng/µl) dNTP mix XL1-Blue super-competent cells pUC18 control plasmid (0.1ng/µl in TE buffer)			
ProteoExtract® Subcellular Proteome Extraction Kit	Wash buffer Extraction buffer I Extraction buffer II Extraction buffer III Extraction buffer IV Protease inhibitor cocktail Benzonase® nuclease	Differential extraction of proteins from mammalian cells based on their subcellular localization	Calbiochem	#539791

Table 17: Devices

Device	Company	Platform
Leica DMI6000B confocal microscope	Leica Microsystems	
LAS AF software version 2.3.0.	Leica Microsystems	
FACSAria™	BD Biosciences	
Flexmap 3D plate reader	Luminex	
Liquid handling	Cybio - An Analytik Jena	

	Company	
VICTOR™ X3 2030 Multilabel Reader	PerkinElmer	
Thermocycler	Peqlab	
GraphPad Prism 5.0	GraphPad	
CellProfiler		The Broad Institute Imaging Platform
Genome-wide human SNP Array 6.0	Affymetrix	
Genotyping console 3.0.2 software	Affymetrix	

Table 18: Antibodies

Antigen	Clone	Use	Conjugate	Company	Catalogue number
Histone H1.2		WB	KLH	Abcam	ab17677
IgG2a		WB		Abcam	
Goat anti-rabbit (secondary)		WB	HRP	Bio-Rad	166-2408EDU
Goat anti-mouse IgG (H+L)		WB	HRP	Bio-Rad	170-6516
AKT		WB		Cell Signaling Technology	#9272
mTOR		WB		Cell Signaling Technology	#2972
Jagged1		WB		Cell Signaling Technology	
Phospho-PDK1 (Ser241)		WB		Cell Signaling Technology	#3061
Phospho-AKT (Thr308)		WB		Cell Signaling Technology	#9275
Phospho-p70 S6 Kinase (Ser371)		WB		Cell Signaling Technology	#9208
Phospho-4E-BP1 (Thr37/46)		WB		Cell Signaling Technology	#9459
V5		IF	AP	Invitrogen	R960-25
Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L)		IF	Alexa-488	Invitrogen	A11001

Alexa Fluor 546 Donkey Anti-Rabbit IgG		IF	Alexa-564	Invitrogen	A10040
PDK1	E3	WB IP		Santa Cruz Biotechnology	sc-17765
GFP	FL	WB	HRP	Santa Cruz Biotechnology	sc-8334 HRP
p53	DO-1	WB	HRP	Santa Cruz Biotechnology	sc-126 HRP
FLAG		WB		Sigma-Aldrich	F7425
MYC	9E10	WB	KLH	Sigma-Aldrich	M4439
V5	V5-10	WB	KLH	Sigma-Aldrich	V8012
HA	HA-7	WB	KLH	Sigma-Aldrich	H9658
Actin		WB		Sigma-Aldrich	A2066
MYC		IF	Maleimide-activated KLH	Sigma-Aldrich	C3956
HSP90		WB		Stressgen	SPS-771
Ras	RAS10	WB		Upstate	#05-516

Table 19: PCR and linear amplification primers for barcode amplification

Primer	Sequence (5'→3')
EPF_New (forward)	CGATTAGTGAACGGATCTC
EPR_2 (reverse)	GAAGGTGAGAACAGGAGC
EPF1-btn (forward)	Biotin-TGAGGATAGCAGAGAAGG

Table 20: Primer sequences for qRT-PCR

Target	Fwd primer (5'→3')	Rev primer (5'→3')
NOTCH3	CCTAGACCTGGTGGACAAG	ACACAGTCGTAGCGGTTG
JAG1	ACACACACTCAGCCTCTGAGGAC	GGGTTTTTGATCTGGTTCAGCT
PIK3CA	ATCTTTTCTCAATGATGCTTGGCT	CTAGGGTCTTTCGAATGTATG
TWIST	TCCGCGTCCCACTAGCA	TTCTCTGGAAACAATGACATCTAGGT
CCNE1	GAGCCAGCCTTGGGACAATA	CGGTCATCATCTTCTTTGTCAGG
AKT1	CATCACACCACCTGACCAAG	CTGGCCGAGTAGGAGAACTG
K-RAS	TAATTGATGGAGAAACCTGTCTCTTG	TTATGGCAAATACACAAAGAAAGCC
IKBKE	TGCGTGCAGAAGTATCAAGC	TACAGGCAGCCACAGAACAG
HDM2	TCTACAGGGACGCCATCGA	CTGATCCAACCAATCACCTGAA
CCND1	GGATGCTGGAGGTCTGCGA	AGAGGCCACGAACATGCAAG
SKP2	TCAACTACCTCCAACACCTATCAC	GACAACTGGGCTTTTGCAGT
TP53	GCCCCCAGGGAGCACTA	GGGAGAGGAGCTGGTGTTG
ZNF217	TCTCAGAACGCATACAGGTGA	CAGCAGCAACATCGGTTTGT
HER2	AACTGCACCCACTCCTGTGT	TGATGAGGATCCCAAAGACC
WIP1	CCCATGTTCTACACCACCAGT	TGGTCCTTAGAATTCACCCTTG
MDM2	TTAGTGGCTGTAAGTCAGCAAGA	CCTTCAGATCACTCCCACCT

TWIST1	CGGGTCATGGCCAACGTG	CAGCTTGCCATCTTGGAGTC
mFGFR1	GCAGAGCATCAACTGGCTG	GGTCACGCAAGCGTAGAGG
BRAF	CAAACTTATAGATATTGCACG	TCTGGTGCCATCCACAAAATG
GSTM1	CTGGGCATGATCTGCTACAATC	CAAAAGTGATCTTGTTTCCTGCAA
c-MYC	CTTCTCTCCGTCCTCGGATTCT	GAAGGTGATCCAGACTCTGACCTT
GAPDH	CGAGCCACATCGCTCAGACA	GGCGCCCAATACGACCAAAT
USP1	TGTTACCATTTGTGGGACTG	CCAGATTTAAAACGGGACA
USP3	CATACCACACCAGAGCCAA	GGCTGCCTTGCCATAAAGCA
USP32	TTCATCCGGGAAGTGCTTGG	TGCAGCCCTTTGGATGTTCC
USP7	ACGAGCCGGACGTTTCGA	GCGCCACTGTGACAAGCATC
USP9x	TCAGGATGTGGGTCGTTACA	TGTCTGCCAAGCCTTTTCTT

Table 21: Primers for site-directed mutagenesis of evolutionary conserved lysine residues of PDK1

Mutant	Fwd primer (5'→3')	Rev primer (5'→3')
K123R	CGACATATCATAAAAGAGAACAGGGTC CCCTATGTAACCAG	CTGGTTACATAGGGGACCCTGTTCTCTTTTA TGATATGTCG
K207R	CATCATTCACAGGGACCTTAGACCGGA AAACATTTTGTTAAATG	CATTTAACAAAATGTTTTCCGGTCTAAGGTCC CTGTGAATGATG
K293/296R	GGAAACGAGTATCTTATATTTCAGAGG ATCATTAGGTTGGAATATGACTTTCCAG	CTGGAAAGTCATATTCCAACCTAATGATCCT CTGAAATATAAGATACTCGTTTCC
K315R	GAGACCTCGTGGAGAGACTTTTGGTTT TAGATGCC	GGCATCTAAAACCAAAAGTCTCTCCACGAGG TCTC
K435R	GTTTTCCGAAGATGAGAGGAGGTTGTT GTTGGAGAAG	CTTCTCCAACAACAACCTCCTCTCATCTTCG GAAAAC
K441R	GTTGTTGTTGGAGAGGCAGGCTGGCG GAAAC	GTTTCCGCCAGCCTGCCTCTCCAACAACAAC
K459R	GAAAATAATTTAATACTAAGGATGGGC CCAGTGGATAAGCGG	CCGCTTATCCACTGGGCCCATCCTTAGTATT AAATTATTTTC
K465/467R	GGGCCCAGTGGATAGGCGGAGGGGTT TATTTGCAAG	CTTGCAAATAAACCCCTCCGCCTATCCACTG GGCCC
K492/494R	GTGGATCCTGTCAACAGAGTTCTGAGA GGTGAAATTCCTTGG	CCAAGGAATTTCACCTCTCAGAACTCTGTTG ACAGGATCCAC
K509R	GAACTTCGACCAGAGGCCAGGAATTTT AAAACTTTCTTTG	CAAAGAAAGTTTTAAAATTCCTGGCCTCTGG TCGAAGTTC

Table 22: Barcode sequences used in Luminex-based genetic and chemical screens

Barcode ID	Sense sequence (5'→3')
1	CTTTAATCTCAATCAATACAAATC
2	CTTTATCAATACATACTACAATCA
3	TACACTTTATCAAATCTTACAATC
4	TACATTACCAATAATCTTCAAATC
5	CAATTCAAATCACAATAATCAATC
6	TCAACAATCTTTTACAATCAAATC
7	CAATTCATTTACCAATTTACCAAT
8	AATCCTTTTACATTCATTACTTAC
9	TAATCTTCTATATCAACATCTTAC
10	ATCATACATACATACAAATCTACA
11	TACAAATCATCAATCACTTTAATC
12	TACACTTTCTTTCTTTCTTTCTTT
13	CAATAAACTATACTTCTTCACTAA
14	CTACTATACATCTTACTATACTTT
15	ATACTTCATTCATTCATCAATTCA
16	AATCAATCTTCATTCAAATCATCA
17	CTTTAATCCTTTATCACTTTATCA
18	TCAAAATCTCAAATACTCAAATCA
19	TCAATCAATTACTTACTCAAATAC
20	CTTTTACAATACTTCAATACAATC
21	AATCCTTTCTTTAATCTCAAATCA
22	AATCCTTTTTACTCAATTCAATCA
23	TTCAATCATTCAAATCTCAACTTT
24	TCAATTACCTTTTCAATACAATAC
25	CTTTTCAATTACTTCAAATCTTCA
26	TTACTCAAAATCTACACTTTTTCA
27	CTTTTCAAATCAATACTCAACTTT
28	CTACAAACAAACAAACATTATCAA
29	AATCTTACTACAAATCCTTTCTTT
30	TTACCTTTATACCTTTCTTTTTAC
31	TTCACTTTTCAATCAACTTTAATC
32	ATTATTCACTTCAAACTAATCTAC
33	TCAATTACTTCACTTTAATCCTTT
34	TCATTCATATACATACCAATTCAT
35	CAATTTCATCATTCATTCATTTCA
36	CAATTCATTTCATTCACAATCAAT
37	CTTTTCATCTTTTCATCTTTCAAT
38	TCAATCATTACACTTTTCAACAAT
39	TACACAATCTTTTCATTACATCAT
40	CTTTCTACATTATTCACAACATTA
41	TTACTACACAATATACTCATCAAT
42	CTATCTTCATATTTCACTATAAAC
43	CTTTCAATTACAATACTCATTACA
44	TCATTTACCAATCTTTCTTTATAC
45	TCATTTCACAATTCAATTACTCAA
46	TACATCAACAATTCATTCAATACA
47	CTTCTCATTAACTTACTTCATAAT

48	AAACAAACTTCACATCTCAATAAT
49	TCATCAATCTTTCAATTTACTTAC
50	CAATATACCAATATCATCATTTAC
51	TCATTTCAATCAATCATCAACAAT
52	TCAATCATCTTTATACTTCACAAT
53	TAATTATACATCTCATCTTCTACA
54	CTTTTTCAATCACTTTCAATTCAT
55	TATATACACTTCTCAATAACTAAC
56	CAATTTACTCATATACATCACTTT
57	CAATATCATCATCTTTATCATTAC
58	CTACTAATTCATTAACATTACTAC
59	TCATCAATCAATCTTTTTCACTTT
60	AATCTACAAATCCAATAATCTCAT
61	AATCTTACCAATTCATAATCTTCA
62	TCAATCATAATCTCATAATCCAAT
63	CTACTTCATATACTTTATACTACA
64	CTACATATTCAAATTACTACTTAC
65	CTTTTCATCAATAATCTTACCTTT
66	TAACATTACAACTATACTATCTAC
67	TCATTTACTCAACAATTACAAATC
68	TCATAATCTCAACAATCTTTCTTT
69	CTATAAACATATTACATTCACATC
70	ATACCAATAATCCAATTCATATCA
71	ATCATTACAATCCAATCAATTCAT
72	TCATTTACCTTTAATCCAATAATC
73	ATCAAATCTCATCAATTCAACAAT
74	TACACATCTTACAAACTAATTTCA
75	AATCATACCTTTCAATCTTTTACA
76	AATCTAACAAACTCATCTAAATAC
77	CAATTAACTACATACAATACATAC
78	CTATCTATCTAACTATCTATATCA
79	TTCATAACTACAATACATCATCAT
80	CTAACTAACAATAATCTAACTAAC
81	CTTTAATCTACACTTTCTAACAAT
82	TACATACACTAATAACATACTCAT
83	ATACAATCTAACTTCACTATTACA
84	TCAACTAACTAATCATCTATCAAT
85	ATACTACATCATAATCAAACATCA
86	CTAATTACTAACATCACTAACAAT
87	AAACTAACATCAATACTTACATCA
88	TTACTTCACTTTCTATTTACAATC
89	TATACTATCAACTCAACAACATAT
90	CTAAATACTTCACAATTCATCTAA
91	TTCATAACATCAATCATAACTTAC
92	CTATTACACTTTAAACATCAATAC
93	CTTTCTATTCATCTAAATACAAAC
94	CTTTCTATCTTTCTACTCAATAAT
95	TACACTTTAAACTTACTACACTAA
96	ATACTAACTCAACTAACTTTAAAC
97	AATCTCATAATCTACATACACTAT

98	AATCATACTCAACTAATCATTCAA
99	AATCTACACTAACAATTTCATAAC
100	CTATCTTTAAACTACAAATCTAAC

Table 23: Hairpin sequences used in Luminex-based genetic and chemical screens

Name	Hairpin sequence	Barcode ID
APC	CCGGTAATGAACACTACAGATAGAACTCGAGTTCTATCTGTAGTGTTCATTATTTTT	10
BRCA1	CCGGGCCTACAAGAAAGTACGAGATCTCGAGATCTCGTACTTTCTTGTAGGCTTTTT	17
BRCA2	CCGGGCTCCACCCTATAATTCTGTTCAAGAGACAGAATTATAGGGTGGAGCTTTTT	2
BRCA2	CCGGGGGAAACACTCAGATTAAAGATTCAAGAGATCTTTAATCTGAGTGTTTCCCTTTTTTT	8
CDKN1B	CCGGGTACGAGTGGCAAGAGGTGTTCAAGAGACACCTCTTGCCACTCGTACTTTTTT	47
CDKN1C	CCGGGCCTCTGATCTCCGATTTCTTCTCGAGAAGAAATCGGAGATCAGAGGCTTTTT	41
CDKN2A	CCGGCATGGTGCGCAGGTTCTTGTTCAAGAGACAAGAACCTGCGCACCATGTTTTTT	38
FBXW7	CCGGCAACAACGACGCCGAATTACTCGAGTAATTCGGCGTCGTTGTTGTTTTTT	20
FBXW7	CCGGGTGTGGAATGCAGAGACTGGAGACTCGAGTCTCCAGTCTCTGCATTCCACACTTTTTT	43
FBXW7	CCGGACAGGACAGTGTTTACAAACTCGAGTTTGTAAACACTGTCCTGTTTTTTT	46
FHIT	CCGGCCCTCTGTAGTGTTTCTCAAACTCGAGTTTGAGAAACACTACAGAGGGTTTTT	26
FHIT	CCGGGCTGGAGACTTTCACAGGAATCTCGAGATTCCTGTGAAAGTCTCCAGCTTTTT	37
PTCH1	CCGGTAATCCTCAACTCATGATACACTCGAGTGTATCATGAGTTGAGGATTATTTTT	45
PTEN	CCGGAAGGCACAAGAGGCCCTAGATTTCTCGAGAAATCTAGGGCCTCTTGTGCCTTTTTTTTT	28
RB1	CCGGCCACATTATTTCTAGTCCAAACTCGAGTTTGGACTAGAAATAATGTGGTTTTT	11
RB1	CCGGACTTCTACTCGAACACGAATCTCGAGATTCGTGTTCGAGTAGAAGTTTTTT	33
SFN	CCGGGGATCCCACTCTTCTTGCACTCGAGTGCAAGAAGAGTGGGATCCTTTTTT	13
SFN	CCGGGTGACCATGTTTCCTCTCACTCGAGTGAGAGGAAACATGGTCACTTTTTT	27
SMAD4	CCGGGCAGACAGAAACTGGATTAAACTCGAGTTTAATCCAGTTTCTGTCTGCTTTTT	15

Table 24: Dharmacon custom library siRNA SMARTpool® sequences targeting human tumour suppressors

siRNA	Target sequence	Duplex catalogue number
ANXA1	CAAAGGUGGUCCCGGAUCA	J-011161-07
	GAAGUGCGCCACAAGCAAA	J-011161-08
	UGACCGAUCUGAGGACUUU	J-011161-09
	UAACUAAGCGAAACAAUGC	J-011161-10
APC	GAUGAUAUGUCGCGAACUU	J-003869-09
	AUGAUAAGCUCCCAAAUAA	J-003869-10
	GAGAAUACGUCCACACCUU	J-003869-11
	GAACUAGAUACACCAAUAA	J-003869-12
BRCA1	CAACAUGCCCACAGAUCAA	J-003461-09
	CCAAAGCGAGCAAGAGAAU	J-003461-10
	UGAUAAAGCUCCAGCAGGA	J-003461-11
	GAAGGAGCUUUCAUCAUUC	J-003461-12
BRCA2	GAAACGGACUUGCUAUUUA	J-003462-05
	GGUAUCAGAUGCUUCAUUA	J-003462-06
	GAAGAAUGCAGGUUUAAUA	J-003462-07
	UAAGGAACGUCAAGAGAUA	J-003462-08
CADM1	CGAAAGACGUGACAGUGAU	J-016565-05
	GUAAUCUGAUGAUCGAUAU	J-016565-06
	AAAGCUCACUCGGAUUAUA	J-016565-07
	GCGCUUGAGUUAACAUGUG	J-016565-08
CDC4	CAACAACGACGCCGAAUUA	J-004264-07
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FHIT	GAAUAGGAAACCUGUGGUA	J-004952-07
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	CUGAUGAAGUGGCCGAUUU	J-004952-09
	CAGGAAGGCUGGAGACUUU	J-004952-10
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	UGACUGUGAUGGCAGGCAA	J-012666-09
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	GUACAGAGUUACUACUUAG	J-003902-12
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	CCGACAUGCUCUCCAAUUU	J-011111-08
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	UGAGAUCGAUGUGGUCAGA	J-008090-10
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	GACCAAGUCUUCCACCAAG	J-007010-08
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	GGCAUCAGUUGUUCAAGUU	J-012386-08

Table 25: Dharmacon ON-TARGETplus™ SMARTpool® siRNA Library-Human Protein Kinase

The catalogue number is # G-103505-01.

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AAK1	GGAAGGUGGAUUUGCUAUU	**MAP3K3**	GAUAGAAGCUCAAGCAUGA
AATK	UAAAGAGGCUUGAGACCUG	**MAP3K4**	CAACAGAGCGUGAUCAUAA
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AATK	GACGACGACUUCCCGCUGA	**MAP3K4**	GAAUAUCAUUGGUCAAGUU
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ABL1	UCACUGAGUUCAUGACCUA	**MAP3K5**	UAAGUAGUCUUCUUGGUAA
ABL1	AGAUAACACUCUAAGCAUA	**MAP3K5**	GCACAAAUACUGAAGAUUC
ABL1	AAGGGAGGGUGUACCAUUA	**MAP3K5**	GGGAAUCUAUACUCAAUGA
ABL1	CAACAAGCCCACUGUCUAU	**MAP3K5**	ACACUACAGUCAGGAAUUA
ABL2	AAUACGAACUCACGGGUAA	**MAP3K6**	CGUGAGAGCUUCAGCAUGA
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ABL2	GGAGUGAAGUUCGCUCUAA	**MAP3K6**	UCAGUGGGCUGCUCAAGAU
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ACVR1	GAAAGGCUGCUUCCAGGUU	**MAP3K7**	GGAAAGCGUUUAUUGUAGA
ACVR1	GUACGACUAUCUUCAGCUU	**MAP3K7**	GCAAUGAGUUGGUGUUUAC
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ACVR1B	GAAACACUUUGACUCCUUU	**MAP3K8**	GACCACCAACGCUUGAAUA
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ACVR1C	GAGGAAUUGUUGAGGAGUA	**MAP3K9**	GAAAUUGUCGUGUAUGAGA
ACVR1C	GACCAUGGCUGGAAUGAUC	**MAP3K9**	CAAACUCGCCCUUCCUAUU
ACVR2	GUUAUCAGCUGGAUGUGUA	**MAP4K1**	GAUACAAUGAGCUGUGUGA
ACVR2	GUUGUUGGCUGGAUGAUAU	**MAP4K1**	CAACAACGUUCUCAUGUCU
ACVR2	GAACUGGCUUCUCGCUGUA	**MAP4K1**	GGAGUUAUCUCUGGUUGCA
ACVR2	CACCUAAGCCACCCUAUUA	**MAP4K1**	GAAAGGACCCUCCAUUGGG
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LAK	GAUGACAGAUCUUAUGUUC
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HAK	GCGAAGACCUUGGCAUUUA
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CSNK1G3	GCUGGGACCUAGUUUGGAA	**PIK3CG**	GACGUCAGUUCCCAAGUUA
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CSNK2A1	GGAAGUGUGUCUUAGUUAC	**PIK3R1**	CCAACAACGGUAUGAAUAA
CSNK2A1	GCUGGUCGCUUACAUCACU	**PIK3R1**	GACGAGAGACCAAUACUUG
CSNK2A1	AACAUUGUCUGUACAGGUU	**PIK3R1**	UAUUGAAGCUGUAGGGAAA
CSNK2A2	GAGUUUGGGCUGUAUGUUA	**PIK3R2**	GCGCCCAGCUUAAGGUCUA
CSNK2A2	GGGACAACAUUCACGGAAA	**PIK3R2**	GGAACGCACUUGGUACGUG
CSNK2A2	GAUAGAUCACCAACAGAAA	**PIK3R2**	GGACAAGAGCCGCGAGUAU
CSNK2A2	UUAAGCAACUCUACCAGAU	**PIK3R2**	GGAAAGGCGGGAACAAUAA
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CSNK2B	GCAAUGAAUUCUUCUGUGA	**PIK3R3**	GAAGGGAGGCAAUAAUAAG
CSNK2B	GCAAGGAGACUUUGGUUAC	**PIK3R3**	GGUCCGAGAUGCCUCAACA
CSNK2B	CAACCAGAGUGACCUGAUU	**PIK3R3**	GAAGAGGACUGCAAUAGAA
DKFZP586B 1621	CGGAAGAGCCAGUUAUAUC	**PIK3R4**	CCAGAAAGGUCCUAUGUUG
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DKFZP586B 1621	GGACGCUCCUUAUCGUGAA	**PIK3R4**	AGUGGUAGCUCGUCAAAUA
DKFZP586B 1621	GGAUAAAGAUGGCAACCGC	**PIK3R4**	CAGCUGAUGUCUACUGUAA
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DAPK2	UUCGGAAGCUUCUGGUUAA	**PIK4CB**	CCUUUAAGCUGACCACAGA
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DAPK2	GGAAUUUGUUGCUCCAGAA	**PIK4CB**	GGACUCACCAGCGCUCUAA
DAPK3	CCACGCGUCUGAAGGAGUA	**PIM1**	GAUGGGACCCGAGUGUAUA
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DCK	CCAGAGACAUGCUUACAUA	**PIP5K1A**	ACACAGUACUCAGUUGAUA

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DDR2	CCAAGUGAUUCUAGCAUGU
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DMPK	GAAAUGUUCUAUGGGCAGA	**PLK4**	GGACCUUAUUCACCAGUUA
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LOC391295	GCCAACAGAGAUUAAGCAA
LOC391533	ACAAGAACGCCGAUUAUGU
LOC391533	CAUCACACAUGGAGCGUAA
LOC391533	GCUCCAAGUUUCAGGAAGA
LOC391533	GCAAACAAAGGGCCUCUGA
LOC391783	GAAACAAGUUAGUACCUUU
LOC391783	GCGAGAAGCUUCAUGACAA
LOC391783	GUGAAGGGUUGAUACUAUA
LOC391783	UGACAAACGUUAUGAGACA
LOC392226	GGAGAUGUCCAUGGGAAUA
LOC392226	UCACGGUCCUCAAUAAACA
LOC392226	AGCCAGAGCUGAUAUUGUC
LOC392226	CCUUGAAGCUGCUACCAUG
LOC392265	ACUAUAAGCUGUACCCAUU
LOC392265	UUACUGAGCUGGAGUGGUC
TK1	GGGCCGAUGUUCUCAGGAA
TK1	GCAUUAACCUGCCCACUGU
TK1	GCACAGAGUUGAUGAGACG
TK1	CAAAGACACUCGCUACAGC
TK2	AAAUCGGGAUCGAAUAUUA
TK2	GUAGAAAACCUGUAUAGAA
TK2	UCACAGCGCAAGAUACAUU
TK2	GAGGAUGUUACAACUCUUU
TLK1	GAGUAUGCAAGAUCGAUUA
TLK1	GAAGCUCGGUCUAUUGUAA
TLK1	GCAAUGACUUGGAUUUCUA
TLK1	GUUCAAAGAUCACCCAACA
TLK2	AAGAUGGCGUGUAGAGAUA
TLK2	UUACAAGGCAUUUGAUCUA
TLK2	GAUAGAAAGACAACGGAAA
TLK2	CCUCAAACCAGGUAAUAUU
TNIK	GAACAUACGGGCAAGUUUA
TNIK	UAAGCGAGCUCAAAGGUUA
TNIK	CGACAUACCCAGACUGAUA
TNIK	GACCGAAGCUCUUGGUUAC
TNK1	GGUCGCACCUUCAAAGUGG
TNK1	GUUCUGGGCCUAAGUCUAA
TNK1	GGCGCAUCCUGGAGCAUUA
TNK1	GGACUUCCUGCGAGAGGUA
TNK2	AAACGCAAGUCGUGGAUGA
TNK2	GAGAACUACUGGUGGCGUG
TNK2	GAAAGCGACUGGAGGCUGA
TNK2	CAUCCUACCUGGAGCGCUA
TNNI3K	UCACACUCCUGAAGCAUUA
TNNI3K	UACAAUAGCUGGCCACCUA
TNNI3K	GGAAUAUGCUCUAAAUGCA
TNNI3K	CUAUGGACAUGAACAGGUA
TP53RK	GGUAGAAGAAUGUGUAUGA
TP53RK	GCUUCCAACUGCUUAUAUA
TP53RK	GCUGAACAUUGUGCUCAUA
TP53RK	GUACCCAUCCCAACACUGA
NYD-SP25	ACUAGCAGCCAAAGAGAGA
NYD-SP25	GGCUGAAAUUGUAACCCUA
NYD-SP25	CCACUCGACUUCUGAACUG
NYD-SP25	GAAGUCGGCCACACUCAGA
TPK1	GCCAACCGCUUAUAUGAUA
TPK1	CGACUCACAUCACUCCUUU
TPK1	UAAGGGAUGUGAGCUCAUU
TPK1	AAGCACAGGUUGCAUGUAG
TRIB1	GCAAGGUGUUUCCCAUUAA
TRIB1	CUAGAAGACACACACAUAA

LOC392265	GCUAAAAGGCCUCGGAUUC
LOC392265	GCCAAUGUAUCUCAGCAGA
LOC392395	CAUAUGAACGGGUCACGUA
LOC392395	GGACUUGGGUUUGGGAGGA
LOC392395	GGAAGAGGCCUGUGAGGUG
LOC392395	GAAAGGCACUGGCUCAAGG
LOC400301	ACUGUGGGCUUAAGUACUU
LOC400301	GGGGCUGGAUUACUGAUUU
LOC400301	CUGCUGGGAUGAAGACAUG
LOC400301	CGAAAGUGGCCUCAUGAAG
LOC401007	GUAUAAGCGUGUGCAUUUA
LOC401007	GACAAGCACUCGACUGAUA
LOC401007	GGACUACAGCCCUUCAGUA
LOC401007	GAAACUGUGUCAGAAUAAG
LOC402269	GCCUUCGGCUGCACAGGUA
LOC402269	GUGAAGCCACCCAGAGUUC
LOC402269	GAGCGUCACCUUCGACAGU
LOC402269	GGACUUCCAUUGUGUUUGA
LOC402353	CAGGCAACGACGCGGAUAA
LOC402353	GCACUGCCCUCCAUGAAGG
LOC402353	GGGCAGGCCUGAGUCUGUA
LOC402353	GACAAUGGCUACCUGCUCU
LOC440332	CCAGAGAAUAUGCGAUUAA
LOC440332	GGAGAAGCGACAUAUCAUA
LOC440332	GAAGGAUACGGACCUCUUA
LOC440332	GGAACAGCGCAGUACGUUU
LOC440451	GAGCGGGCAAUGAUAGUAA
LOC440451	GGGCGAGACCCUCCAACAA
LOC440451	GAGAAUACACAGUACACGA
LOC440451	GCACGCUCCUCCAAGCUUG
LOC440785	GCCGGAGUCUUAUAUUUAA
LOC440785	GGAAACCCUAGCUAUAUUG
LOC440785	UGAAGCAGCUGUCCAAGCA
LOC440785	GAUUAUGGCUUCAUGACAG
LOC441435	UGGAAUGAUUGCAACGUUA
LOC441435	GCACGUGGGCGACAGUCAA
LOC441435	GAAGGGCGAUGUACUCGGC
LOC441435	GAGAUGCGAAGAAGAGGUA
LOC441777	ACGAAGGUCUCGCGACAUU
LOC441777	GAUCUGGACUGGCAACCAA
LOC441777	CAAGAUCGCACCUGAGUCA
LOC441777	GGGUGGGACUCCUGAUUAU
LOC441787	GAACAGACUUUGUAACAGU
LOC441787	CGCCAGAGGUUGCUUACUG
LOC441787	CAAGUGAAUUCGGAUCCUU
LOC441787	GUUAAACUCUGGGACCGUU
TRIB1	CGGAAAGGCUGCGGACGUU
TRIB1	GAACCCAGCUUAGACUAGA
TRIB2	UCGAAGAGUUGUCGUCUAU
TRIB2	CGGCUGACCUCGCAGGAAA
TRIB2	CAUAGUAACAUCAACCAAA
TRIB2	UGUGCAAGGUGUUUGAUAU
TRIB3	CUACGUGGGACCUGAGAUA
TRIB3	CAACUUAGAUACCGAGCGU
TRIB3	GAAACGAGCUCGAAGUGGG
TRIB3	GCACUGAGUAUACCUGCAA
TRIO	GUAAAGAAGUGAAAGAUUC
TRIO	CGACCUAUCCGUAGCAUUA
TRIO	GGAAUACAACCACGAAGAA
TRIO	AGAACAGGGUAUUGCAUUA
TRPM6	GAGAGGAGGCCACGGUUAA
TRPM6	CGAAUAACCUUGAUUGACA
TRPM6	CUAAUACACUCUUGCAUCA
TRPM6	GCUGAAGGGCACAAAUUUA
TRPM7	GUUAAUACAUGGUCAAGUA
TRPM7	UCAGUUGGCCGUUGAAUUA
TRPM7	GACCAAAGAUUGAUACAGU
TRPM7	CCAUAUUGCUGUUAGAGUA
STK22D	CCAGCAAGCCGUCGACUAU
STK22D	GGAUGACAGUGGUCGAAUG
STK22D	GGACGAAGCUCGCAAGAAG
STK22D	GAAGUUCAAUGUGGCGAUC
STK22B	GCAAGGGUUCCUACGCAAA
STK22B	GAAGAAGGGUUACAUCGUA
STK22B	GGACGGAUCUACAUCAUCA
STK22B	GCAAGCACCUAGCAUGACA
STK22C	GAAGUUAGUUGGCAUCCAU
STK22C	GGAGUCUGCCGACGGGAAA
STK22C	GAUAUGAUCCUCCGGCCUU
STK22C	GGGAGGGCCAGAAGAGUUU
C14ORF20	ACACAAAGCAGAAGGUUAU
C14ORF20	GCUCCUAUGGGUCGGUAUA
C14ORF20	GGAUGAAUAUGGUUAUGAG
C14ORF20	CUAAACAGCACCAAUCCUU
SSTK	UGAGCGAACUCGGUUAUAA
SSTK	UCCAAGAAGUACAAGGGUA
SSTK	GGGAAACUGUACAUCGUGA
SSTK	AGAAGUACGAUGUGUGGAG
TTBK1	GAACAGGUAGGGAUGAUCA
TTBK1	GGCAGGAACGAGAAGUUUA
TTBK1	GAAUGAGGCCUUUGACUGG
TTBK1	CGUCAUGUCUUCCGGUGGA

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LOC442313	GCUCCCAGCUGUAGCCUCA
LOC442313	CUACAGCAGUCUUUAUGGA
LOC442313	GAGCACAAGGGCCCAAUUC
LOC442402	UAAGAGCGAUACUGGUGAA
LOC442402	GCACUGCCCUCCAUGAAGG
LOC442402	GACAAUGGCUACCUGCUCU
LOC442402	GAGCGAUCUCUGCACUUAU
LOC91461	GAAGCGGAACCUCUAUAAU
LOC91461	UCACAUACCUCCUGCCUCA
LOC91461	CCAACAAGACCACAUAUGU
LOC91461	GGAGCCAAGUGGUCCCUGA
LRRK1	GAAUGGUGCUCUACGAGUU
LRRK1	GGACGCACCUGGAUUUAAU
LRRK1	GGACACAUGCUCACCCAAA
LRRK1	GAUCAGGCCUCGCAUUGUA
LRRK2	GAAAUUAUCAUCCGACUAU
LRRK2	GGAGGGAUCUUCUUUAAUU
LRRK2	UUACCGAGAUGCCGUAUUA
LRRK2	CAAGUUAUUUCAAGGCAAA
LTBP1	GGAGGGUACUAACUGCAUA
LTBP1	GGGAGUGCCUAAACACAGA
LTBP1	AAUGGUGAGUGUUUGAAUA
LTBP1	GAUGACCUGUGUCGAUGUA
LTBP4	CAUAUGGGCCUGAGUUGUA
LTBP4	CGAGUACGGCCCAGACUUA
LTBP4	CGAGUGCGCCGAUGAGGAA
LTBP4	GUGCAAGAGUGGCGUGUGU
LTK	CCACGGAGAGGUAGAGAUC
LTK	GAACUCUGCUCGCCUCAGG
LTK	GGACUUCGUCGUUGGAGGA
LTK	GGGAACAGAUCUUUGGAGU
LYK5	CUGUGAAUCUAGCAAGGUA
LYK5	GGAUUAACCUAGAAGCUUG
LYK5	UGUGAUAGGCAAAGGAUUU
LYK5	UGAAUGAGCUGGCGAUUGC
LYN	GCGACAUGAUUAAACAUUA
LYN	GUGAUGUUAUUAAGCACUA
LYN	GAGAUCCAACGUCCAAUAA
LYN	UUACAUCUCUCCACGAAUC
BAIAP1	GGACGUGGCUUCAAAUAGU
BAIAP1	GAAGCGGGAAACACAGUUA
BAIAP1	GGACGCCAGUAAUUGGAAA
BAIAP1	GAAAGAAGGGGAUCUCAUA
MAGI-3	GUGACUAUCCCUUUGAUUA
MAGI-3	UACCAGGAGUGGAUUAUAA
TTBK2	GACCAUGUUUGUAGAUUUA
TTBK2	UGGCUUGGCUCGACAAUUU
TTBK2	GGGCAGCAUUGUAUUGAGA
TTBK2	GACCAUAUCUCUUCUUUGG
TTK	GAUAAGAUCAUCCGACUUU
TTK	GCAAUACCUUGGAUGAUUA
TTK	CCAGUUAACCUUCUAAAUA
TTK	GAUAGUUGAUGGAAUGCUA
TTN	GUGGAGACAUUACAUAUUA
TTN	GGUACUCAAUCACUAAUUA
TTN	GAAUUGAUGUCCUGUGGAA
TTN	UGUGGAAGCUCGUCAUAUA
TXK	GGCAGAACAUCUAUUGAGA
TXK	GCUGAUGGGCAGUUGUUUA
TXK	CGUGGAAGCUAUUUCUGAA
TXK	GCACCAAUGUCCAUAUAUG
TYK2	GCACAAGGACCAACGUGUA
TYK2	CAAUCUUGCUGACGUCUUG
TYK2	GAGAUCCACCACUUUAAGA
TYK2	GCAAGCCUGAUGCUAUAUU
TYRO3	ACGCUGAGAUUUACAACUA
TYRO3	GCGAUGAACUAAAGGAAAA
TYRO3	CAGUGUGGCUCACGGUAGA
TYRO3	GCGCAUCGAGGCCACAUUG
UCK1	GGAAGUCGACCGUGUGUGA
UCK1	GAACAUCGUGGAGGGCAAA
UCK1	GGAGGGACCUGGAGCAGAU
UCK1	CGACAAAGAAGUAUGCCGA
UMPK	UACGAGACCUGUUCCAGAU
UMPK	CCGCAGACGUGGUGCUCUU
UMPK	GAUCAUCCCUAGAGGUGCA
UMPK	CAGGAUAGCUUCUACCGUG
URKL1	CGGGUUAGCUGCCCAGUGA
URKL1	GCGACGAGUUCAUCUUCUA
URKL1	CAAGCAAGCGUACCAUCUA
URKL1	GAACACGGCACGCAAUCCA
UHMK1	CGAGUAUGGUUUCCGCAAA
UHMK1	GAUGUUUGAUGGGAAGUUU
UHMK1	GUUCUGCUAUUAUUGAUCA
UHMK1	UCAAACCACGUAACAUAUU
ULK1	CAGCAUCACUGCCGAGAGG
ULK1	CCACGCAGGUGCAGAACUA
ULK1	GCACAGAGACCGUGGGCAA
ULK1	UCACUGACCUGCUCCUUAA
ULK2	GUGGAGACCUCGCAGAUUA
ULK2	UCAGACCACUCAUGUGAUA

MAGI-3	AGAUAGACCUGAUGAGUUC
MAGI-3	CCUGACCAGUCUAUAUAUA
MAK	CAAGUGAGGUCGAUGAAAU
MAK	GGACGUACUUAUAAUCCUA
MAK	UCAAGUUGGUCAGGUAUUA
MAK	UGACCGAAAUGUUGAAUUG
MAP2K1	CCAUGCUGCUGGCGUCUAA
MAP2K1	GAGGUUCUCUGGAUCAAGU
MAP2K1	CGACGGCUCUGCAGUUAAC
MAP2K1	GCACAAGGUCCUACAUGUC
MAP2K1IP1	CGGAUGACCUAAAGCGAUU
MAP2K1IP1	GAGAUGGAGUACCUGUUAU
MAP2K1IP1	CUAAUUGUCAGCCUAGAAA
MAP2K1IP1	GGAACUUGCUCCAUUGUUU
MAP2K2	CGACAGCGCAUGCAGGAAC
MAP2K2	GAUCAGCAUUUGCAUGGAA
MAP2K2	GGUCCGAGGUGGAAGAAGU
MAP2K2	UCUUUGAACUCCUGGACUA
MAP2K3	CCGCAGAGCGUAUGAGCUA
MAP2K3	GGAGAUUGCUGUGUCUAUC
MAP2K3	CAGAAGGGCUACAAUGUCA
MAP2K3	UGGACAAGUUCUACCGGAA
MAP2K4	CCAUGUAUGUCGAUUGAUA
MAP2K4	GUAAUGCGGAGUAGUGAUU
MAP2K4	UAACACAAGUCGUGAAAGG
MAP2K4	CCACAGGCCGAUUUCCUUA
MAP2K5	CCUCCAAUAUGCUAGUAAA
MAP2K5	UAAGUGCGAUUCAUCAUAU
MAP2K5	GGAGCCAUUUGUACAUUUC
MAP2K5	GAAUUGCAGUAGCAGUUGU
MAP2K6	CCAAAGAACGGCCUACAUA
MAP2K6	CGUCAAGCCUUCUAAUGUA
MAP2K6	GAUAAAGGCCAGACAAUUC
MAP2K6	GAUCCGAGCCACAGUAAAU
MAP2K7	CAAAGACUGCCUUACUAAA
MAP2K7	UCAUGAAGCAGACGGGCUA
MAP2K7	GCAUUGAGAUUGACCAGAA
MAP2K7	UCAAGGAUGUCAUGGCGAA
MAP3K1	GAUUAGAUGUCAAUACAGA
MAP3K1	GACUAAGAAUUGCAGAUUU
MAP3K1	ACAUUUGUCUCCUGGUUUA
MAP3K1	GUGGAGAUCUCAUGAUUUC
MAP3K10	GGACCGCGGUGUUCGACUA
MAP3K10	CAACAUAAUUGCCCUUAGG
MAP3K10	UAGAGGAGAUCAUCGGUGU
MAP3K10	UGAACUACCUACACAAUGA
ULK2	CGAUAUAAAUUCUGCAUCA
ULK2	UCCAAGAUCUGCAGUGGUA
DKFZP434C131	GCACGUACGCCACGGUGUA
DKFZP434C131	CGACCUGUCUCGCUUCAUC
DKFZP434C131	CAUAAAGUGUGUAGCCAAG
DKFZP434C131	CUGAGAAGGUGGCGCGUGU
ULK4	CCUCGUAACUUCACAGGUA
ULK4	CCACGGUUGUUGACUAUAU
ULK4	GUAUCGGCCUUGUGUAGAA
ULK4	GUAAUACCAUGCAAAGUGU
VCPIP1	GAGAAGCUCUGGUGAUUAU
VCPIP1	GGGACAGACUUUAGUAAUA
VCPIP1	GGAGAUGGGUCUAUUGUGU
VCPIP1	CGACAGAAUUACAAUAGAA
VRK1	GCAGUUGGAGAGAUAAUAA
VRK1	AUACUUGGUUAUUGCAUGA
VRK1	GGCUUUGGCUGUAUAUAUC
VRK1	AGGUGUACUUGGUAGAUUA
VRK2	GCAAUUAGGUAUCCGAAUG
VRK2	CACAAUAGGUUAAUCGAAA
VRK2	GGAAUAGAUUUACAGAAGA
VRK2	CCAGAAUGGUACCUUUAAA
VRK3	CCAAGCGGCAUUCAAAUUC
VRK3	CUGCAUGGGUUUCGGUGUU
VRK3	GGACAGUGCUGACAGACAA
VRK3	CCACCUCACUUGAAGCUUU
WEE1	AAUAGAACAUCUCGACUUA
WEE1	AAUAUGAAGUCCCGGUAUA
WEE1	GAUCAUAUGCUUAUACAGA
WEE1	CGACAGACUCCUCAAGUGA
WIF1	GUACGAAGCCAGCCUCAUA
WIF1	CAGCACACGCCUUCACUUA
WIF1	GUGCCGAAAUGGAGGCUUU
WIF1	GGCAUCAGUUGUUCAAGUU
PRKWNK1	GCAGUUGUCUCAAUAUCUA
PRKWNK1	GCAGGAGUGUCUAGUUAUA
PRKWNK1	UAAAGGAGACCGAGUAGUA
PRKWNK1	GAGAGUCGUUUGUGGAUCA
PRKWNK2	GGACGCACCCGAUGAAAUU
PRKWNK2	CGUAUAUGGUGGAGCAUGA
PRKWNK2	GCAAACCUCGUGCUCGAUU
PRKWNK2	CCACGAGAGUGACGUCAAG
PRKWNK3	GAUUGGUGAUCUAGGAUUA
PRKWNK3	CCACAUCGGAGUAUCCUUA
PRKWNK3	UCUGAAAGGUUGUCUAUCA
PRKWNK3	GGACUAAAUUUCAGCUUAC

MAP3K11	CAUGGUACCUGGAUUCAGA	**WNK4**	CGGGCACGCUCAAGACGUA
MAP3K11	UGGCGUAGCUGUUAACAAG	**WNK4**	CAGCUGCCAUGGUAUAUAA
MAP3K11	CUCCAAAGGCAGCGGGUUA	**WNK4**	GUUCUCAGGUCACUCUUAG
MAP3K11	GGAGAAACGUCUUCGAGGU	**WNK4**	GCGGGUCACUAGUGUCUCA
MAP3K12	GGACAUUGCCUCAGCUGAU	**XYLB**	GGUAAAGGUUGUUGCUGUU
MAP3K12	GAACAUGCGCCAGUCACUA	**XYLB**	CAGCAACACGGAAGUAUAU
MAP3K12	GAACUUAAUGCCCUCAUGU	**XYLB**	GUUCUGGAAUGAAUUUGUU
MAP3K12	GAAAGCCACUCGUAUUCCU	**XYLB**	GAGGUUCGAGCACUAAUUG
MAP3K13	GAUCACACCUCGAUUGCUA	**YES1**	GCAAAGGGAACAGCAGUUA
MAP3K13	AAGCGGAACUGAAGACAUA	**YES1**	GAUCUUCGGGCUGCUAAUA
MAP3K13	GGAUAUUCGUGAACACUAU	**YES1**	GGAAAGUAUUUGAAGCUUC
MAP3K13	AGGCAUAUUCCACUGAUUA	**YES1**	CAGAAGACCUUUCAUUUAA
MAP3K14	GGAUUGACCUCACCCAGAA	**FLJ23074**	GUAGUGGGCUCAGGAUAUA
MAP3K14	GAACCGGGCACUACAGCAA	**FLJ23074**	GAGAUGGUGUUCUGUAAAU
MAP3K14	GUCCAAAUACAGUCUCUUA	**FLJ23074**	GACAGAAGGUGUUGAGAUC
MAP3K14	GCCAGUGGAUUAUGAGUAC	**FLJ23074**	GGACGGAAAUCAGAUAUCU
MAP3K15	GUUAUACACUUUCGGAUAU	**ZAK**	CGAGAAAGACGUUUAAAGA
MAP3K15	CCAAGGGCCUCGCGGAUAU	**ZAK**	GGAUAUCACAGGACAAGGA
MAP3K15	UCAGAGAACGGCUACAUUA	**ZAK**	GAGUGGAGGUGCGAAAUUG
MAP3K15	GAAAGGCACGUAUGGGAUU	**ZAK**	CAAGAAACGUUGUUAUAGC
MAP3K2	GUAAUAAUGAUGGUCGUAA	**ZAP70**	GCAACGUCCUGCUGGUUAA
MAP3K2	GAAGAGAAUUGGCUGUUAA	**ZAP70**	CCUCAUAGCUGACAUUGAA
MAP3K2	GUACACAGGCUACUAAUUU	**ZAP70**	GAACUGUACGCACUCAUGA
MAP3K2	GAACAUAUCCAAGAAGGUA	**ZAP70**	GGAGAUCCCUGUGAGCAAU

Table 26: siRNA sequences

siRNA	Target sequence	Company	Duplex catalogue number
c-MYC	CGAUGUUGUUUCUGUGGAA	Dharmacon	LU-003282
(SMARTpool)	AACGUUAGCUUCACCAACA		
	GAACACACAACGUCUUGGA		
	ACGGAACUCUUGUGCGUAA		
Luciferase	UCGAAGUAUUCCGCGUACG	Dharmacon	D-002050-01-20

Table 27: Compounds used in the Luminex-based chemical screen

Drug name	100x stock concentration (ng/µL)
(s)BI-2536 TsOH Salt	200
ABT-737	200
ABT-869	200
AC220	200
Actinomycin D	0.2
AG13958	20
Akt-I-1,2	200
Akt-I-1	2000
AMG-Tie2-1	20

AS-25242	200
AT7519	200
AT9283	2
AV-412	0.2
AZD6244	2
BEZ235	2
BI-D1870	2
BMS-2	200
BX795	20
BYK204165	20
CC-401	200
CI-1033	2
CI-1040	2
CP-690550	2000
CP-724714	200
CYC-116	2
CYT11387	2
Dasatinib	0.2
E7080	200
Erlotinib-HCl	2
Flavopiridol	0.2
Gefitinib	2
GW441756	200
HP-470	2000
Imatinib-Mesylate	200
JNJ-38877605	200
JNJ-7706621	2
KI20227	200
KU0063794	2
KU55933	200
Lapatinib	2
Masatinib	2
Merck-5	2
Nilotinib	20
Nocodazole	0.2
NU1025	2000
Pazopanib	20
PD0325910	2
PD-04217903	200
PD173955-Analogue 1	20
PD173955	200
PF431396	0.2
PF562271	2
PI103	2

PI93	20
PIK-75	0.2
PIK-90	2
PLX4720	200
Purvalanol B	200
R1487	2000
Rho-15	200
RWJ-67657	200
SB202190	200
SB203580	200
SB216763	200
SB242235	200
SB590885	200
SNS-032	0.2
SNS-314	200
SR3677	200
SU-5402	200
SU6668	200
Sunitinib	20
TAK-715	200
Tandutinib	200
TG100115	200
TG101209 Derivative 1	2
TG101209 Derivative 2	2
TG101209	2
TG101348	20
TGX221	2000
Vandetanib	2
VX-680	2
VX-702	2000
YM201636	20
Z-LLL-AL	0.2
ZSTK474	2
DMSO	0.25%

Table 28: Sequence of PDK1, ubiquitin B, GFP and peptide and protein tags

PDK1 DNA sequence (*Homo sapiens*)
ATGGCCAGGACCACCAGCCAGCTGTATGACGCCGTGCCCATCCAGTCCAGCGTGGTGTTATGTTCCTGCCCAT
CCCCATCAATGGTGAGGACCCAGACTGAGTCCAGCACGCCCCCTGGCATTCCTGGTGGCAGCAGGCAGGGCC
CCGCCATGGACGGCACTGCAGCCGAGCCTCGGCCCGGCGCCGGCTCCCTGCAGCATGCCCAGCCTCCGCCG
CAGCCTCGGAAGAAGCGGCCTGAGGACTTCAAGTTTGGGAAAATCCTTGGGGAAGGCTCTTTTTCCACGGTTGT
CCTGGCTCGAGAACTGGCAACCTCCAGAGAATATGCGATTAAAATTCTGGAGAAGCGACATATCATAAAAGAGA
ACAAGGTCCCCTATGTAACCAGAGAGCGGGATGTCATGTCGCGCCTGGATCACCCCTTCTTTGTTAAGCTTTAC

TTCACATTTCAGGACGACGAGAAGCTGTATTTCGGCCTTAGTTATGCCAAAAATGGAGAACTACTTAAATATATTC
GCAAAATCGGTTCATTCGATGAGACCTGTACCCGATTTTACACGGCTGAGATTGTGTCTGCTTTAGAGTACTTGC
ACGGCAAGGGCATCATTCACAGGGACCTTAAACCGGAAAACATTTTGTTAAATGAAGATATGCACATCCAGATCA
CAGATTTTGGAACAGCAAAAGTCTTATCCCCAGAGAGCAAACAAGCCAGGGCCAACTCATTCGTGGGAACAGCG
CAGTACGTTTCTCCAGAGCTGCTCACGGAGAAGTCCGCCTGTAAGAGTTCAGACCTTTGGGCTCTTGGATGCAT
AATATACCAGCTTGTGGCAGGACTCCCACCATTCCGAGCTGGAAACGAGTATCTTATATTTCAGAAGATCATTAA
GTTGGAATATGACTTTCCAGAAAAATTCTTCCCTAAGGCAAGAGACCTCGTGGAGAAACTTTTGGTTTTAGATGC
CACAAAGCGGTTAGGCTGTGAGGAAATGGAAGGATACGGACCTCTTAAAGCACACCCGTTCTTCGAGTCCGTCA
CGTGGGAGAACCTGCACCAGCAGACGCCTCCGAAGCTCACCGCTTACCTGCCGGCTATGTCGGAAGACGACGA
GGACTGCTATGGCAATTATGACAATCTCCTGAGCCAGTTTGGCTGCATGCAGGTGTCTTCGTCCTCCTCCTCAC
ACTCCCTGTCAGCCTCCGACACGGGCCTGCCCCAGAGGTCAGGCAGCAACATAGAGCAGTACATTCACGATCT
GGACTCGAACTCCTTTGAACTGGACTTACAGTTTTCCGAAGATGAGAAGAGGTTGTTGTTGGAGAAGCAGGCTG
GCGGAAACCCTTGGCACCAGTTTGTAGAAAATAATTTAATACTAAAGATGGGCCCAGTGGATAAGCGGAAGGGT
TTATTTGCAAGACGACGACAGCTGTTGCTCACAGAAGGACCACATTTATATTATGTGGATCCTGTCAACAAAGTT
CTGAAAGGTGAAATTCCTTGGTCACAAGAACTTCGACCAGAGGCCAAGAATTTTAAAACTTTCTTTGTCCACACG
CCTAACAGGACGTATTATCTGATGGACCCCAGCGGGAACGCACACAAGTGGTGCAGGAAGATCCAGGAGGTTT
GGAGGCAGCGATACCAGAGCCACCCGGACGCCGCTGTGCAGTGA

PDK1 amino acid sequence (*Homo sapiens*)

MARTTSQLYDAVPIQSSVVLCSCPSPSMVRTQTESSTPPGIPGGSRQGPAMDGTAAEPRPGAGSLQHAQPPPQPRK
KRPEDFKFGKILGEGSFSTVVLARELATSREYAIKILEKRHIIKENKVPYVTRERDVMSRLDHPFFVKLYFTFQDDEKLY
FGLSYAKNGELLKYIRKIGSFDETCTRFYTAEIVSALEYLHGKGIIHRDLKPENILLNEDMHIQITDFGTAKVLSPESKQA
RANSFVGTAQYVSPELLTEKSACKSSDLWALGCIIYQLVAGLPPFRAGNEYLIFQKIIKLEYDFPEKFFPKARDLVEKLL
VLDATKRLGCEEMEGYGPLKAHPFFESVTWENLHQQTPPKLTAYLPAMSEDDEDCYGNYDNLLSQFGCMQVSSSS
SSHSLSASDTGLPQRSGSNIEQYIHDLDSNSFELDLQFSEDEKRLLLEKQAGGNPWHQFVENNLILKMGPVDKRKGL
FARRRQLLLTEGPHLYYVDPVNKVLKGEIPWSQELRPEAKNFKTFFVHTPNRTYYLMDPSGNAHKWCRKIQEVWRQ
RYQSHPDAAVQ

Ubiquitin B (*Homo sapiens*)

ATGGGCGCACCCTGTCTGACTACAACATCCAGAAAGAGTCCACCCTGCACCTGGTGCTCCGTCTCAGAGGTGG
G

GFP

TTGGGGTTGCGCCTTTTCCAAGGCAGCCCTGGGTTTGCGCAGGGACGCGGCTGCTCTGGGCGTGGTTCCGGG
AAACGCAGCGGCGCCGACCCTGGGTCTCGCACATTCTTCACGTCCGTTCGCAGCGTCACCCGGATCTTCGCCG
CTACCCTTGTGGGCCCCCCGGCGACGC

HA-tag

YPYDVPDYA

His-tag

HHHHHH

FLAG-tag

DYKDDDDK

MYC-tag

EQKLISEEDL

V5-tag

GKPIPNPLLGLDST

Strep-tag II

WSHPQFEK

Table 29: Deubiquitinase cDNA library

Screen ID	DUB name
1	USP26
2	USP30
3	ATXN3
4	OTUD4
5	OTUD5
6	OTUD6B
7	OTUD7B
8	YOD1
9	BRCC3
10	EIF3S3
11	EIF3S5
12	PARP11
13	PSMD7
14	PSMD14
15	COPS5
16	COPS6
17	USP42
18	USP43
19	USP44
20	USP45
21	USP53
22	USPL1
23	UCHL3
24	UCHL5
25	BAP1
26	USP28
27	USP29
28	USP42
29	USP33
30	USP36
31	USP37
32	USP38
33	USP3
34	USP5
35	USP7

Screen ID	DUB name
36	USP8
37	USP10
38	USP11
39	USP12
40	USP13
41	USP14
42	USP15
43	USP16
44	USP18
45	OTUB1
46	USP1
47	USP2
48	USP39
49	USP46
50	USP48
51	USP49
52	USP50
53	USP52
54	CYLD
55	UCHL1
56	STAMBPL1
57	TNFAIP3
58	VCPIP1
59	JOSD2
60	OTUB2
61	USP20
62	USP21
63	USP22
64	USP25
65	DUB3
66	STAMBP
67	JOSD1
68	JOSD3
69	USP19
70	USP4

4.2 Methods

4.2.1 Cell culture

The human cell lines were obtained from the American Type Culture Collection. MCF10A (fibrocystic, non-tumourigenic) were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 5% horse serum (Gibco), 1% penicillin/streptomycin, insulin (10µg/ml), cholera toxin (100ng/ml), EGF (20ng/ml) and hydrocortisone (500ng/ml) (Sigma-Aldrich). HEK293T (human embryonic kidney cells), HeLa (cervix carcinoma), U2OS (osteosarcoma), HaCat (keratinocytes), A549 (alveolar basal epithelial carcinoma), SK-HEP1 and HUH7 (hepatoma), MCF7 (breast adenocarcinoma), BT474 (breast ductal carcinoma), HCC70 (breast ductal carcinoma) and BT549 (breast ductal carcinoma) cells were maintained in DMEM supplemented with 10% fetal calf serum (Gibco) and 1% penicillin/streptomycin at 37°C, 5% CO_2 and 95% humidity. K562 (chronic myelogenous leukemia), A2780 (ovarian carcinoma), THP-1 (acute monocytic leukemia), SNU-387 and HepG2 (hepatoma) were cultured in RPMI-1640 medium with 10% fetal calf serum and 1% penicillin/streptomycin. All cells were incubated at 37°C, 5% CO_2 and 95% humidity.

4.2.2 SNP array analysis

Genetic variations in MCF10A cells were monitored by genome-wide human SNP array 6.0 (Affymetrix) according to manufacturer's instructions using hg18 genome assembly and genotyping console 3.0.2 software (Affymetrix).

4.2.3 Barcoded vectors and isogenic cell line generation

The stuffer fragment in the lentiviral vector pLKO.1 (Moffat et al, 2006) was replaced with a short linker sequence and barcodes (Luminex-designed FlexMap barcode tags (24 bp), Table 22, page 173) flanked by primer sites and inserted 5′ of the U6 promoter. This so-called pLKO.2 vector was then used to introduce stable DNA barcodes into cells by lentiviral transduction.

Briefly, the 5' NotI site in pLKO.1-stuffer was destroyed and oligos containing the barcode cloning site was ligated into the now unique NotI site 5' from the U6 promoter. Inserted verified sequence was: GGCCCGGGGCTAGCTGAGGATAGCAGAGAAGGACGCGTGATCCTGCAGCTCC

TGTTCTCACCTTCGCGGCCGCTCTAGAGTCGACTACGTACGGGGC. A second sequence containing various restriction sites was cloned 3' from the stuffer into the EcoRI site, leaving the 5' site intact. Inserted verified sequence was: AATTCTACGTAGATATCTGATCACCATACGTATGGCCCGGGT. The stuffer sequence was removed and a short linker sequence containing a RNA polymerase III termination sequence was inserted: TGATCTTTTTGATCGATC.

Barcode sequences were cloned into MluI/PstI sites. Cloning oligos into pLKO.2 using the AgeI and EcoRI restriction sites generated short hairpin RNA expressing vectors. An overview of hairpin sequences used in this study is illustrated in Table 23 (page 175).

HeLa and MCF10A isogenic cell lines overexpressing cDNAs or shRNAs were produced by retro- or lentiviral transduction, respectively, and subsequent selection. Stable lines were cultured for approximately 4 to 6 weeks prior to the screen and barcoded by a second infection when needed. Prior to tumour suppressor siRNA SMARTPool transfections (Dharmacon, Table 24, page 176) MCF10A were infected with barcoded lentivirus.

Retro- and lentiviral particles were produced by $CaPO_4$ method in HEK293T producer cells. 7 x 10^5 cells per well were seeded in a 6-well plate in 2ml growth medium 24 h prior transfection and transfected with 0.23µg of plasmid VSV-G, 1.77 µg of plasmid pPol (retrovirus production) or dR8.91 (lentivirus production), 0.2µg plasmid CMV-GFP as transfection control and 1µg vector of interest. Total 3.2µg of DNA were diluted in 158µl HEPES buffered saline (HBS; see Table 14, page 164) and 8.3µl 2.5M $CaCl_2$ were added while vortexing. Transfection mixture was added to HEK293T cells. Medium was replaced by 1ml fresh growth medium 24 h post-transfection. Viral supernatants were collected 36, 48 and 60 h post-transfection, pooled and centrifuged at 2000 rpm for 5 min at 4°C. 20-100% viral supernatant mixed with 7µg/ml polybrene (Sigma-Aldrich) were used to spin infect 60% confluent cells in a 6-well format by centrifuging target cells at 2000 rpm for 30 min at 30°C. Transduced cell lines were either selected using 2µg/ml puromycin (puro, Sigma-Aldrich), 500µg/ml neomycin (neo, Invitrogen) or 100µg/ml hygromycin (hygro, Invitrogen) respectively, or sorted by fluorescence activated cell sorting (FACS, BD FACSAria™) for GFP expression or infected for up to eight rounds to ensure infection of more than 90% of cells, respectively. Derivatives of pBABE, pMIG, pQXp, MDH1-PGK-GFP and pLNCX vectors were used for retrovirus production. Derivatives of pLMJ, pEF, pLKO.1 and pLKO.2 served for lentivirus production.

4.2.4 Luminex-based screening set-up

For each compound a 4-point dose-response curve was obtained in the parental MCF10A cells using the CellTiter-Glo assay (Promega). The corresponding luminescence signal was measured for 1 second per well with VICTOR^{TM} X3 2030 Multilabel Reader (PerkinElmer). Cellular viability was normalized to DMSO control and plotted against drug concentration. These results defined compound concentrations used in the screen (see Table 27, page 214). All barcoded cell lines were pooled, counted and distributed in multi-well plates in quadruplicate. With the help of Cybio liquid handling robot, compound or DMSO control was added 16 h after seeding. Growth medium was refreshed every second day. Cells were split once during a total of nine days after which genomic DNA was isolated and barcode amplification was performed by PCR.

For the genetic synthetic lethal interaction screens (cancer gene vs. kinome), all barcoded isogenic cells expressing a distinct cDNA or shRNA were pooled, counted and distributed in multi-well plates in quadruplicate. Each kinase siRNA (Dharmacon #G-103505-01, Table 25, page 180) was added at a final concentration of 50nM 16 h after seeding. Cells were cultured for a total of seven days after which genomic DNA was isolated and barcodes were amplified by PCR.

Genomic DNA extraction was performed with the Cybio liquid handler using the Genfind v2.0 kit (Agencourt). Briefly, growth medium was removed and cells were washed twice with phosphate buffered saline (PBS). After lysis (see Table 14, page 164), 100µl raw lysate was transferred into 96-deep-well plates and 60µl of the Agencourt binding buffer were added. Beads were washed six times with 70% ethanol and purified genomic DNA was eluted in dH_2O. Barcodes were amplified by PCR using GoTaq® PCR kit (Promega) in a two-step protocol (synthesis cycle: 5 min at 95 °C, 12 x (3 min at 95 °C, 1.5 min at 54 °C, 2 min at 72 °C), 22 x (30 sec at 95 °C, 30 sec at 54 °C, 30 sec at 72 °C), 10 min at 72 °C) and linear amplification was performed with a 5′ biotinylated primer (synthesis cycle: 5 min at 95 °C, 40 x (30 sec at 95 °C, 30 sec at 54 °C, 30 sec at 72 °C), 10 min at 72 °C). The primers used at 200nM each are listed in Table 19 (page 171). The single-stranded product was hybridized to pre-coupled Luminex xMAP beads (Stegmaier et al, 2007). Briefly, 15µl of microspheres (1.2 x 10^4 microspheres/µl in Tris-EDTA buffer (TE buffer; see Table 14, page 164) were mixed with 3µl single-stranded product and 34µl 1.5 x TMAC buffer (see Table 14, page 164). After 2 min incubation at 94°C, barcode

hybridization was allowed for 1.5 h at 40°C in 384-well plates. Hybridized product was incubated with streptavidin-coupled phycoerythrin (SAPE, Invitrogen; 2.5ng/µl in 1x TMAC (see Table 14, page 164) for 30 min at 40°C. Beads were washed once and resuspended in 50µl TE buffer. Fluorescence intensity of samples was measured in a Flexmap 3D plate reader (Luminex) at 40°C.

4.2.5 Luminex dataset analysis

Raw signal intensities of barcode-hybridized beads were determined for each barcode (i.e. gene) and well (i.e. drug treatment). The multiple measurements for each barcode in each well were replaced by their medians. The barcode median values from each well were normalized by a factor such that their sum became identical in all the wells. All the normalized medians were log-transformed. A first robust linear regression was computed according to the additive model data ~well+barcode+residual (Wilkinson-Rogers notation), where “data” is representative for the log-transformed normalized medians, “well” stands for the well-specific deviation, “barcode” represents the barcode-specific deviation and “residual” is the residual data variability. This regression model yielded the final data normalization step by independently removing the well and barcode systematic biases. The residuals were then further subjected to a robust linear regression to decipher the well-barcode interactions. This second regression model includes all the possible combinations of barcode and well effects (factor crossing) to explain the remaining residuals residual ~well×barcode+error. The p-values of these second regression coefficients were used to rank the interactions, whereas the regression coefficients themselves were indicative for the magnitude of the well-barcode interactions. A detailed and advanced outline of the algorithm will be published elsewhere (Muellner et al, 2013).

4.2.1 Statistical analysis

Statistical significance and distribution of the data obtained (Kolmogorov-Smirnov test) were calculated in GraphPad Prism 5.0 (GraphPad software). Experiments were performed in triplicate unless otherwise noted. $P<0.05$ (alpha=5%) was considered statistically significant.

4.2.2 Resistance scores calculation

Resistance scores were calculated by first excluding all normalized data points in the sensitivity direction. The fold change (drug vs. all) was then calculated for each drug treatment of ICN1, c-MYC and MDM2 as a control gene. The mean resistance scores were then calculated by taking all PI3K inhibitor data (minus BEZ-235) or all remaining drugs.

4.2.3 Oncomine analysis

The Wooster cell-line dataset describes over 300 cell lines (GSK300) with their respective profiles for gene expression, copy number (CHG) and sensitivity to 19 compounds including the PI3K/mTOR inhibitors BEZ-235, GSK1059615, temsirolimus and the Aurora kinase inhibitor GSK1070916 (Greshock et al, 2010). The analysis was performed by grouping the drugs based on target pathway such as PI3K/mTOR or Aurora kinase. A c-MYC copy number >4 was indicative for c-MYC gene amplification and the resistant/sensitive classification and median expression of NUMB were used according to Oncomine.

4.2.4 GFP competition assay

After transduction with cDNAs for ICN1 or GFP (EF-hICN1-CMV- GFP), respectively, or an empty control vector, cells were pooled, seeded in multiple 6-well plates and subjected to BEZ-235 or DMSO as control treatment. GFP-positive cells were detected by FACS or a Leica DMI6000B microscope. For the microscopy analysis, ten randomly chosen fields were captured for each cell line-drug combination. Cells were quantified by CellProfiler (The Broad Institute). Uninfected cells served to measure background fluorescence levels.

4.2.1 Crystal violet staining

Cells were washed with PBS, fixed with 3.7% formaldehyde in PBS for 30 min and stained with 0.1% crystal violet in 5% ethanol in PBS overnight followed by extensive washing with PBS.

4.2.1 Quantitative real-time PCR

Total RNA was extracted from sub-confluent cells using Trizol (Invitrogen) or RNeasy kit (Qiagen) according to manufacturer's protocol. Samples were subjected to DNase treatment (Turbo-DNA free, Ambion) prior cDNA synthesis. Briefly, RNA was incubated with 5µl of 10 x TURBO DNase buffer and 1µl DNase in a total of 50µl reaction volume for 20-30 min at 37°C. The treatment was stopped by incubating with 5µl DNase Inactivation Reagent at room temperature, while mixing occasionally. The RNA sample was collected by centrifuging at 13.000 rpm for 1.5 min. 0.1ng-5µg RNA was incubated with 1µl random hexamer primers (Fermentas) for 5 min at 65°C. Reverse transcription was performed using 20 units/µl RiboLockRNase Inhibitor, 10mM dNTP mix, 200 units/µl RevertAid M-MuLV Reverse Transcriptase (Fermentas) according to manufacturer's instructions (synthesis cycle: 10 min at 25°C, 60 min at 42°C and 10 min at 70°C). Quantitative real-time PCR (qRT-PCR) was carried out using the iTaq SYBR Green Supermix (Bio-Rad) (5µl SYBR Green, 10pmol/µl primer mix, 1µl cDNA in 10µl total reaction volume) (synthesis cycle: 2 min at 50°C, 10 min at 95°C, 40 x (15 sec at 95°C, 1 min at 60°C)). Measurements were performed in triplicate and related to GAPDH as a reference gene. All the primer sequences used are listed in Table 20 (page 171).

4.2.2 Immunoprecipitation, pull-downs and immunoblotting

For immunoprecipitation and PIP3 (Echelon Biosciences) pull-down, cell extracts were prepared in RIPA buffer (see Table 14, page 164) or in ELB buffer (see Table 14, page 164) supplemented with protease inhibitors (Complete, Roche) and *N*-Ethylmaleimide (Sigma-Aldrich). The lysate was sonicated for 2 x 6 seconds on ice, centrifuged at 14.000 rpm for 10 min at 4°C and pre-cleared with 10µl Protein A/G agarose beads (Pierce #20421). Immunoprecipitation was performed with 20µl Protein A/G agarose bead suspension and 1µg antibody per 1ml lysate or pre-coupled agarose bead suspension (anti-HA agarose, Sigma-Aldrich A2095; anti-V5, Sigma-Aldrich A7345).

For isolation of His-tagged proteins, cells were lysed under denaturing conditions (see Table 14, page 164) supplemented with *N*-Ethylmaleimide and sonicated. Lysates were incubated for 4 hours with nickel beads (Sigma-Aldrich H0537) and washed with imidazole buffer (see Table 14, page 164). Beads were resuspended in 4 x SDS sample buffer (see Table 14, page 164) and boiled for 3 min at 95°C.

For immunoblotting, proteins were resolved by 4-12% Bis-Tris polyacrylamide gels (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in 2% i-Block (Applied Biosystem) or 4% bovine serum albumin (BSA) for 20 min and probed with the appropriate antibody overnight at 4°C. Detection of bound antibodies was performed by incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies at room temperature for 1 h followed by ECL according to the manufacturer's instructions (ECL-PLUS, GE Health Care; Pierce ECL, Thermo Scientific).

The antibody list is summarized in Table 18 (page 170). Anti-PDK1 (sc-17765), anti-GFP (sc-8334) and anti-p53 (sc-126) were purchased from Santa Cruz. Anti-FLAG (F7425), anti-MYC 9E10 (M4439), anti-V5 V5-10 (V8012), anti-HA HA-7 (H9658) and anti-Actin (A2066) were obtained from Sigma-Aldrich. Anti-phospho-specific S241 PDK1 (#3061) and T308 AKT (#9275) were purchased from Cell Signaling Technology. Anti-HSP90 (SPS-771) and Anti-H1.2 (ab17677) were obtained from Stressgen and Abcam, respectively.

4.2.3 Compounds

Rapamycin, everolimus, mitomycin C, PP242 and LY294003 were purchased from Sigma-Aldrich. All other compounds were obtained from SynThesis Medchem (China). Compound purity was ~95% according to the manufacturer except for PP242 (~98%). J. Bradner (Dana-Farber Cancer Institute) has kindly provided the gamma secretase inhibitor dibenzazepine. Mass spectrometry analysis and published standards validated the compound purity and identity.

4.2.4 Plasmids and cell transfection

4.2.4.1 Luminex-based screen

siRNA experiments in MCF10A cells were performed by transfecting 10nM siRNA using siLentFect lipid reagent (Bio-Rad) according to manufacturer's instructions. The recently described mTOR-targeting shRNA was obtained by cloning oligos into pLKO.1 (Sarbassov et al, 2005). The construct was verified by Sanger sequencing.

4.2.4.2 PDK1 and ubiquitin

The pcDNA6-PDK1-V5-His vector was generated by cloning a PCR fragment with EcoRI and XbaI restriction sites (Fwd primer 5'GAATTCGCCAGGACCACCAGCCAG3', Rev primer 5'TCACTGCACAGCGGCGTCTCTAGA3') into the pcDNA6 backbone. All other mutants were generated by site-directed mutagenesis using the Stratagene Dpn1 PCR protocol. Evolutionary conserved lysines residues of PDK1 and the primer sequences used in site-directed mutagenesis into alanine are summarized in Table 21 (page 172).

His-tagged ubiquitin expression vector was created by cloning human ubiquitin B into pcDNA3 using the restriction sites BamHI and XbaI. The PDK1-ubiquitin C-terminal fusion protein was created by PCR amplifying human ubiquitin B and cloning it 3' of PDK1 in pcDNA6-PDK1-V5-His vector using KpnI and EcoRI.

PDK1 kinase domain (KD) expression vector was created by PCR amplification and cloning into pcDNA6-V5-His using EcoRI and XbaI. The following primers were used: Fwd primer 5'-GATCGAATTCCCACCATGGCCAGGACCACCAGC-3', Rev primer 5'-GATCTCTAGACGGGTGAGCTTCGGAGGCGTC-3'.

HA-tagged ubiquitin expression vector (pRK5-HA-ubiquitin wild type) and the 68 DUB expression vectors harbouring both FLAG and HA tags were obtained from Addgene. cDNAs encoding the full-length USP19-WT, USP4-WT and -C311S proteins in the pcDNA4-MYC-His vectors were kindly provided by Dr. Kristina Lindsten. USP4-WT and -C311S cDNAs were subcloned into pcDNA6-V5-His. DUBs used in the screen are listed in Table 29 (page 218).

The lysine-less PDK1 mutant was ordered from Mr.Gene (Regensburg, Germany).

All generated constructs and mutants were verified by Sanger sequencing.

Transfections were performed using $CaPO_4$ method or with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Cells were harvested between 48 and 72 hours post-transfection.

4.2.5 BigDye Sequencing

BigDye sequencing was performed using 250ng plasmid according to manufacturer`s instructions. The following cycles were used: 1 min at 96°C, 25 x (10 sec at 96°C, 5 sec at 50°C, 4 min at 60°C), hold at 4°C. A 250µl G50 sephadex bead (5% in H_2O) column was used for BigDye purification. BigDye reaction sample was mixed with 10µl formamide on a 96-well PCR plate and heated 2 sec at 98°C to denature strands prior to Sanger sequencing.

4.2.6 Subcellular Proteome Extraction

Cell fractionation was performed using the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem #539791) according to manufacturer's protocol. Briefly, potentially contaminating media components were removed by extensively and carefully washing the cells with ice cold washing buffer. Subcellular fractions were enriched using individual extraction buffers: buffer I for cytosolic proteins, buffer II for membrane and membrane organelles, buffer III for nucleus and cytoskeleton, buffer IV for cytoskeleton.

4.2.7 Inducible PDK1 expression

4.2.7.1 Gateway cloning

Expression vectors used for retroviral transfection (PDK1-TEV-Myc-ProtG) and Flip recombination were generated by the gateway cloning system. This site-specific two-step recombination method consists of inserting attB flanked DNA of interest (PCR product) into an entry clone (EC) vector (BP reaction) which can then be transferred to a destination vector (DV) using recombinases specific for att recombination sites (LR reaction).

attB-PDK1-attB was PCR amplified from pcDNA6-PDK1-V5-His (Fwd primer: GGGGACAAGTTTGTACAAAAAAGCAGGCTCCGCCAGGACCACCAGCCAG, Rev primer: GGGGACCACTTTGTACAAGAAAGCTGGGTTCACTGCACAGCGGCGTC).

For the BP reaction, the EC was generated by recombination of the PCR product with pDONR backbone vector, whereby 1µg pDONR, 3µg PCR DNA product, 2µl BP clonase and 4µl dH_2O were mixed. After 1 h incubation at room temperature, 1µl

Proteinase K was added to stop the reaction and samples were incubated for 10 min at room temperature. Ligation product was transformed into XL10-Gold competent bacteria according to the manufacturer's protocol (Agilent Technologies). Briefly, 1-2µl DNA mixed with 25µl competent cells were incubated on ice for 30 min. Cells were then heat-shocked 20 sec at 42°C and immediately cooled on ice 2 min. After incubation in LB medium without antibiotic for 30 min at 37°C, cells were subjected to kanamycin selection on LB agarose plates. Resistant colonies were grown up in LB medium with kanamycin 30µg/ml. Plasmid DNA preparation was carried out according to the manufacturer's protocol (Qiagen). The insert was verified by Sanger sequencing.

For the LR reaction, 3µg EC, 1µg destination vector, 2µl LR clonase and 4µl dH_2O were mixed. After 1 h incubation at room temperature, 1µl Proteinase K was added to stop the reaction and samples were incubated for 10 min at room temperature.

4.2.7.2 Flp-In recombination system

PDK1 wild type cDNA sequence was cloned into an expression vector with both StrepIII and HA sequences using the gateway cloning system. The so-called HEK293TSH cell line, which inducibly expresses StrepIII-HA-tagged PDK1 (STREP-HA-PDK1), was then generated by Flp-In recombination. Flp-In T-Rex 293 cells (Invitrogen) were transfected with 200ng LR (pcDNA5/FRT/TO/SH/GW-PDK1) construct and 1µg pOG44 (recombinase) vector using siLentFect (Bio-Rad) according to manufacturer's protocol. Transfected cells were subjected to Blasticidin Hydrochloride (38µg/ml) and Hygromycin B (260µg/ml) selection for one week. The maintenance of colonies was achieved by changing media every three days under selection with Blasticidin Hydrochloride (15µg/ml) and Hygromycin B (100µg/ml) for approximately 2-3 weeks. Expression of STREP-HA-PDK1 was induced with 1µg/ml doxycycline for 24 hours, while uninduced cells were used as a control. This system has been developed based on standard tandem affinity purification (TAP) protocols to enhance throughput and yield in large-scale proteomics studies (Glatter et al, 2009). The method allows stable expression of PDK1 at comparable rates in different experiments after doxycycline induction. It also enables culturing cells in larger amounts for lysis. A further advantage is that the StrepIII-tag interacts strongly with the streptavidin derivative Strep-Tactin (Vasilescu & Figeys, 2006).

For Streptavidin/Strep-Tactin purification, lysate was prepared in RIPA buffer and

sonicated 2 x 6 seconds on ice, centrifuged at 14.000 rpm for 10 min at 4°C. Precipitation was achieved with 400µl Strep-Tactin sepharose packed column, washed twice with lysis buffer. Elution was done using a 2.5mM D(+)-Biotin solution in lysis buffer. Strep-Tactin is a streptavidin derivative engineered to bind the 8 amino acid peptide Trp-Ser-His-Pro-Gln-Phe-Glu-Lys (StrepII tag) via its biotin-binding pocket (Schmidt & Skerra, 2007). StrepIII tag is sequential arrangement of StrepII tags with a poly-Gly and poly-Ser linker (Junttila et al, 2005). The subsequent mass spectrometry analysis was performed by Sylvie M Noordermeer and Bert A van der Reijden at Radboud University Nijmegen Medical Centre, Laboratory of Hematology, Nijmegen 6525 GA, Netherlands.

4.2.8 *In vitro* deubiquitination assay

V5-tagged USP4 was immunoprecipitated with anti-V5 beads and co-incubated with purified STREP-HA-PDK1 overnight at 30°C in a final volume for 15µl of deubiquitination buffer (see Table 14, page 164). The reaction mixtures were analysed by immunoblotting with the anti-PDK1 antibody.

4.2.9 Confocal microscopy

U2OS cells were seeded on glass coverslips in 6-well dishes and transfected with 4µg cDNA using Lipofectamine 2000 according to manufacturer's protocol. 48 hours post-transfection, cells were fixed with 4% paraformaldehyde at room temperature for 15 min, permeabilized with 0.1% Triton X-100, blocked with 3% BSA and incubated with anti-V5 (Invitrogen R960-25) or anti-MYC 9E10 (Sigma-Aldrich C3956). Cells were washed and incubated with Alexa-488- (Invitrogen A11001) and Alexa-564-conjugated (Invitrogen A10040) secondary antibodies or 4,6-diamidino-2-phenylindole (DAPI; 2µg/ml) for 1 h. Coverslips were mounted onto slides with ProLong Gold (Molecular Probes). Immunofluorescence of stained cells was visualized with a Leica DMI6000B confocal microscope. Images were captured with LAS AF software version 2.3.0.

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Education and Career

2013-present	Institute of Pharmacology and Toxicology, Department of Biomedical Sciences, University of Veterinary Medicine Vienna, Austria Group of **Univ. Prof. Dr. Veronika Sexl** Title: "Identification of cell cycle related targets in BCR-ABL driven neoplasms"
International PhD Program	
2008-2012	Research Center for Molecular Medicine of the Austrian Academy of Sciences (CeMM), Medical University of Vienna, Austria Group of **Dr. Sebastian Nijman** Title: "USP4 inhibits the mono-ubiquitination of PDK1" Second project: "Synthetic Lethality Screen: Finding the genotype-specific Achilles' Heels of Human Cancer"
Master thesis	
2006-2007	Research Institute of Molecular Pathology (IMP), Vienna, Austria Group of **Univ. Prof. Dr. Erwin F. Wagner** Title: "Interaction of the proto-oncogene *c-jun* and the tumour suppressor gene $p19^{ARF}$ in cell cycle progression

and cancer development"

2001-2008 Master of Science study program in Molecular Microbiology at the University of Vienna with exceptional graduation

Bachelor program in Biology -Genetics and Microbiology- at the University of Vienna

1993-2001 Österreichisches St. Georgs-Kolleg, Naturwissenschaftliches Realgymnasium, Istanbul, Turkey with exceptional graduation

Scholarship

2001-2007 Awarded by "Alumni Foundation of St. George Austrian High School" for exceptional graduation from high school; Grade: 1 (1: excellent; 5: unsatisfactory)

Scientific Experiences

June-September 2002 **Istanbul University Cerrahpasa Medicine Faculty: Experimental Animal Breeding and Research Laboratory**

1) The protective effects of coenzyme-Q 10 on the toxic stress in rats

2) The antioxidant activity of lycopene in rats

2001-2007 **University of Vienna:** Practical Trainings

Teaching Experiences

2009-present Assistant in "Chemistry (gesunde und kranke Menschen/ SOL-selbstorganisiertes Lernen)" at the Medical University of Vienna

Supervisor of a master student and an intern at CeMM

2003-2008 Tutor in "Experimental Physics", "Experimental

Chemistry", at the practical course in "Learning the microorganisms" at the University of Vienna

Leadership Experiences

2011-2012 Board member and workshop coordinator at Young Scientist Association (YSA) of the Medical University of Vienna

2011-2012 PhD student representative at CeMM

2000 **Global Young Leaders Conference (GYLC)**
in Washington D.C. and New York City, USA

Organized Lectures

2012 YSA Workshop "Surviving in Science"
8th YSA PhD Symposium

2010-2011 Constantin Spiegelfeld Lecture Series at CeMM
as a board member

Others

2011 Excel-Workshop at CeMM

2010 Workshop "Patent and Intellectual Property Protection and Management" at CeMM

2008 Workshop "CyBi-Well Robotics" at CeMM

2001-2003 Internship and freelancer at the newspaper Hurriyet in Istanbul, Turkey

Attended conferences, posters and presentations

Posters:

2011 Cold Spring Harbor Laboratory "The Ubiquitin Family":
"A novel post-translational modification on PDK1"
Cold Spring Harbor, New York, USA

2010 — 6th PhD Symposium of Young Scientist Association of Medical University of Vienna:
"Synthetic Lethality Screen: Finding the genotype-specific Achilles' Heels of Human Cancer"
Vienna, Austria

2009 — 5th PhD Symposium of Young Scientist Association of Medical University of Vienna:
"Synthetic Lethality Screen: Finding the genotype-specific Achilles' Heels of Human Cancer"
Vienna, Austria

2008 — ISREC EPFL LSS08 "Cancer and the Cell Cycle": "Functional Interaction between *c-jun* and $p19^{ARF}$ in cell cycle regulation and cancer development"
Lausanne, Switzerland

2003 — 5th International Veterinary Medicine Students Scientific Research Congress: "The antioxidant activity of lycopene at rats: Examining whether lycopene prevents damages caused by alcohol to the liver"
Istanbul, Turkey

Oral presentations:

2011 — 7th PhD Symposium of Young Scientist Association of Medical University of Vienna:
"A novel post-translational modification on PDK1"
Vienna, Austria

Workshop and Congress:

2012 — 3rd European Chemical Biology Symposium "SMALL MOLECULES FOR BIG BIOLOGY"
Vienna, Austria

2012 — Symposium on Personalized Medicine
Vienna, Austria

2011 EMBO Workshop “Synthetic Lethality from Yeast to Man”
Vienna, Austria

2010 Planet xMAP - Multiplexing Congress
Vienna, Austria

Prizes

2011 Best oral presentation in Oral Session 5 at the 7th PhD Symposium of Medical University of Vienna

Publications

2013 PAK-dependent STAT5 serine phosphorylation is required for BCR-ABL-induced leukemogenesis

Berger A*, Hoelbl-Kovacic A*, Bourgeais J, Hoefling L, Warsch W, Grundschober E, **Uras IZ**, Menzl I, Putz EM, Hoermann G, Schuster C, Fajmann S, Leitner E, Kubicek S, Richard Moriggl R, Gouilleux F, Sexl V
Leukemia (submitted) (* equal contribution)

2013 Ataxia Telangiectasia Mutated (ATM) mutations in lung cancer are synthetic lethal with MEK inhibitors

Smida M*, Fece de la Cruz F*, **Uras IZ**, van Jaarsveld R, Muellner MK, Haura EB, Loizou JI, Nijman SM
Nature (submission) (* equal contribution)

2013 TOPS: A versatile software tool for statistical analysis and visualization of combinatorial gene-gene and gene-drug interaction screens

Muellner MK*, Duernberger G*, Ganglberger F*, Kerzendorfer C, **Uras IZ**, Schonegger A, Bagienski K, Colinge J, Nijman SM
BMC Bioinformatics (submitted) (*equal contribution)

2012 Ubiquitin-specific protease 4 inhibits mono-ubiquitination of the master growth factor signaling kinase PDK1

Uras IZ, List T, Nijman SM
PLOS ONE. 2012;7(2):e31003. doi: 10.1371/journal.pone.0031003.

2011 A chemical-genetic screen reveals a mechanism of resistance to PI3K inhibitors in cancer

Muellner MK, **Uras IZ**[*], Gapp BV[*], Kerzendorfer C, Smida M, Lechtermann H, Craig-Mueller N, Colinge J, Duernberger G, Nijman SM
Nat Chem Biol. 2011 Sep 25;7(11):787-93. doi: 10.1038/nchembio.695. ([*] equal contribution)

2009 Cardiac glycosides induce cell death in human cells by inhibiting general protein synthesis

Perne A[*], Muellner MK[*], Steinrueck M, Craig-Mueller N, Mayerhofer J, Schwarzinger I, Sloane M, **Uras IZ**, Hoermann G, Nijman SM, Mayerhofer M
PLOS ONE. 2009 Dec 16;4(12):e8292. doi:10.1371/journal.pone.0008292. ([*] equal contribution)

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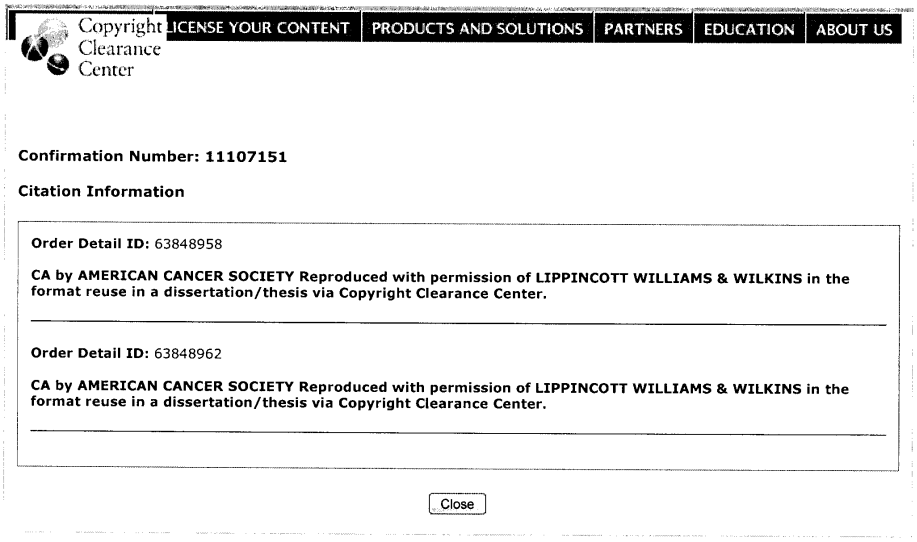

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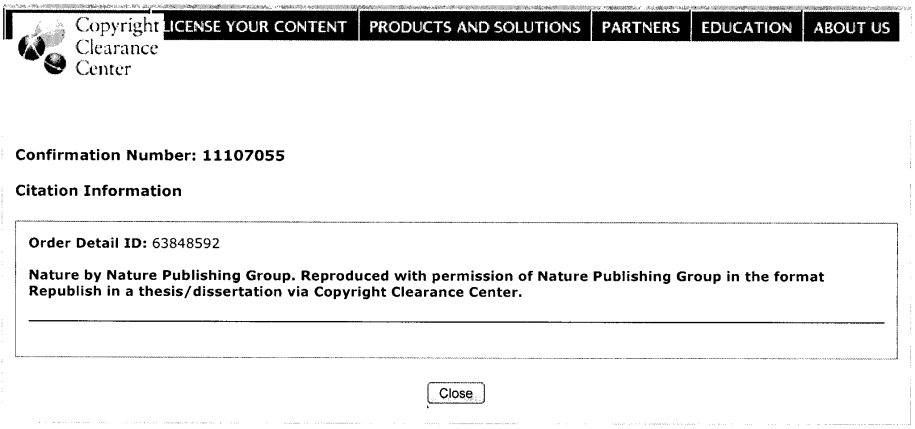

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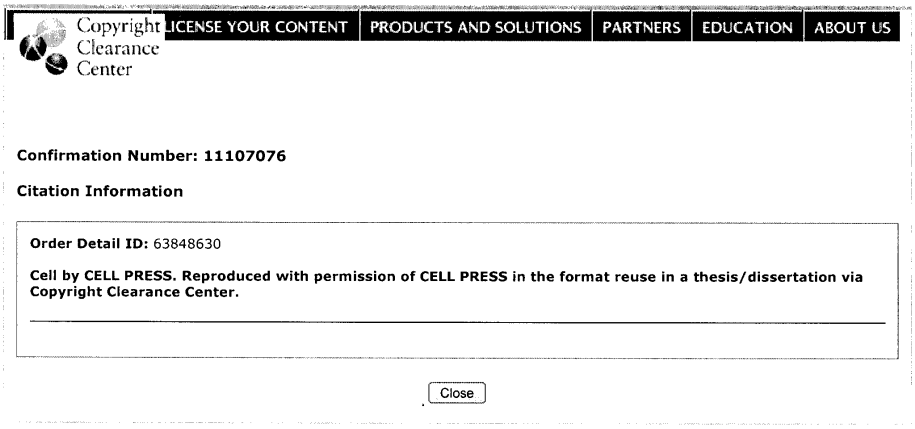

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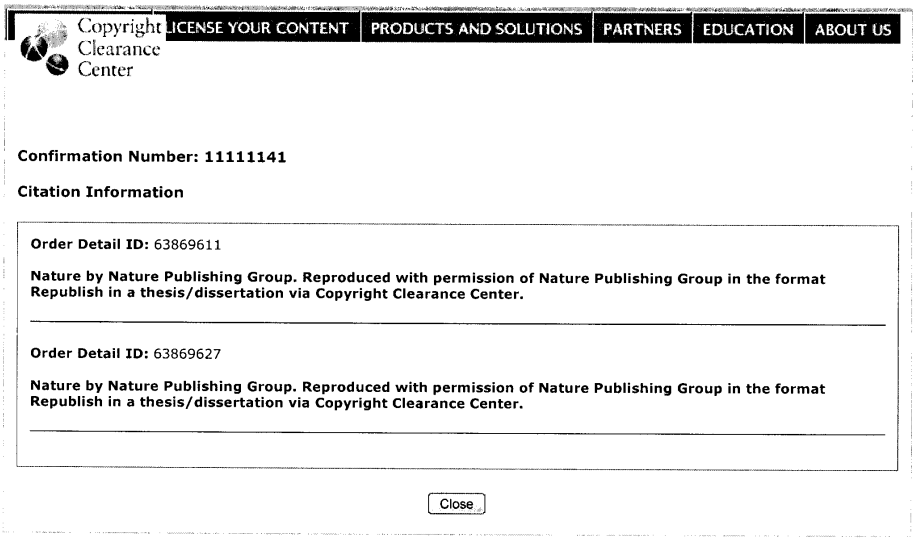

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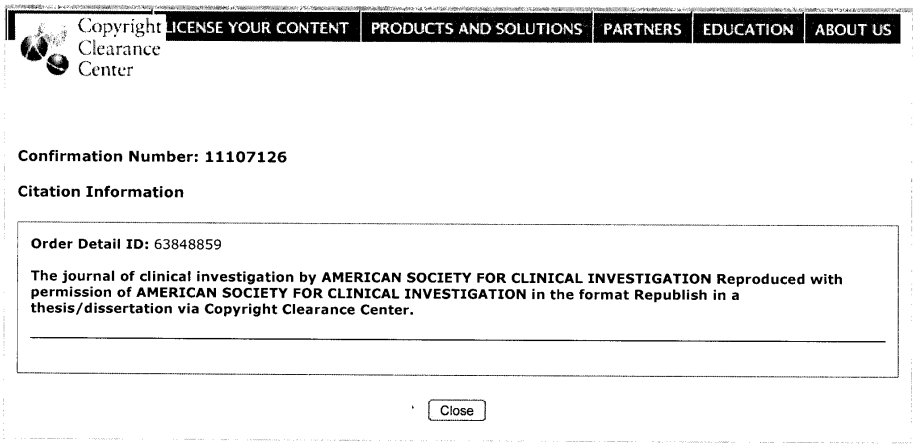

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Annals of oncology : official journal of the European Society for Medical Oncology

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Order detail ID:	63848872
Article Title:	What can breast cancer molecular sub-classification add to conventional diagnostic tools?
Author(s):	Andre, F ; Domont, J ; Delaloge, S
DOI:	10.1093/ANNONC/MDM291
Date:	Jul 01, 2007
ISSN:	0923-7534
Publication Type:	Journal
Volume:	18
Issue:	Supplement 9
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Expected completion date	Nov 2013
Estimated size(pages)	300
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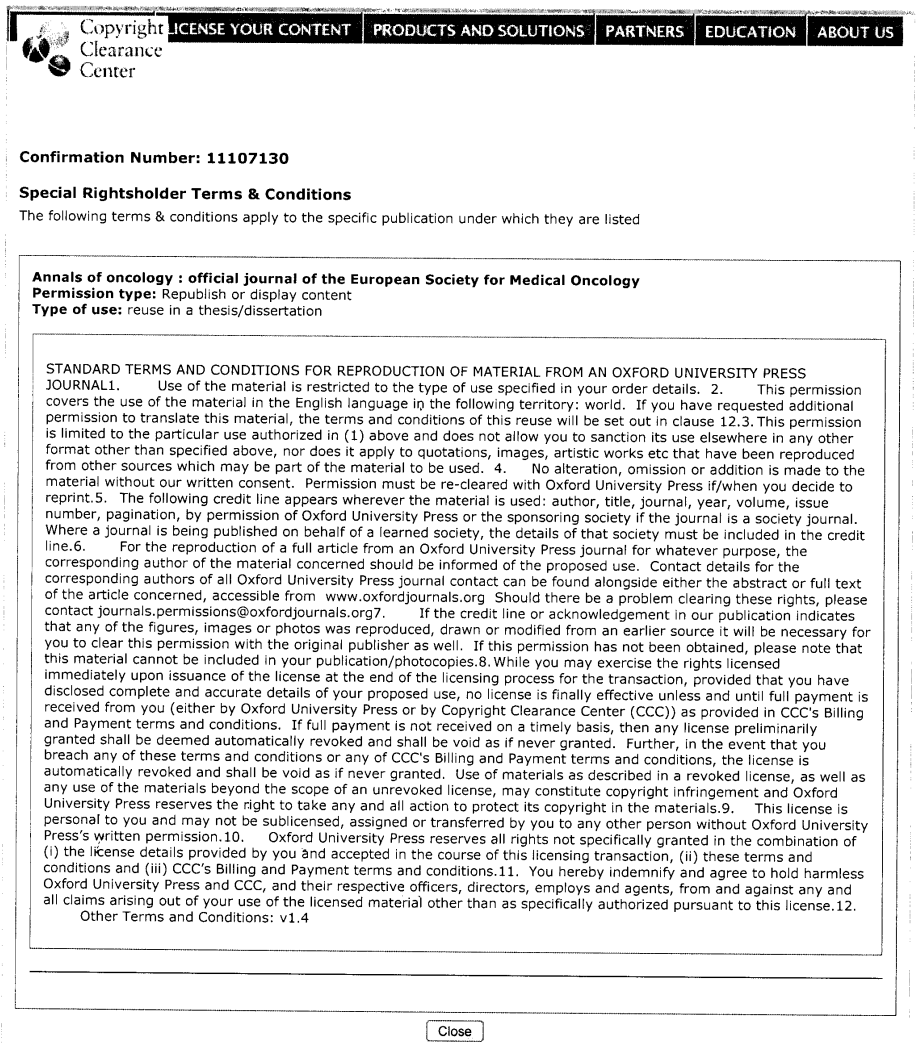

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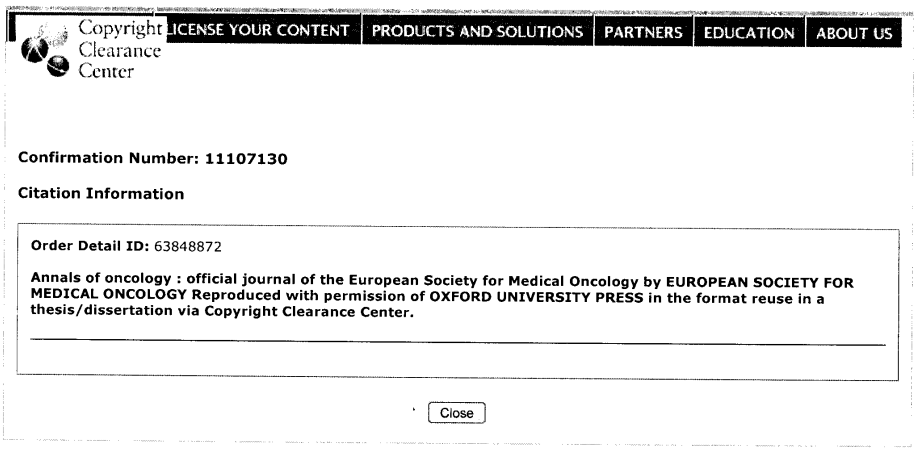

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Article Title:	Identifying Breast Cancer Druggable Oncogenic Alterations: Lessons Learned and Future Targeted Options
Author(s):	Ocana, A. ; Pandiella, A.
DOI:	10.1158/1078-0432.CCR-07-1630
Date:	Feb 15, 2008
ISSN:	1078-0432
Publication Type:	Journal
Volume:	14
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Start page:	961
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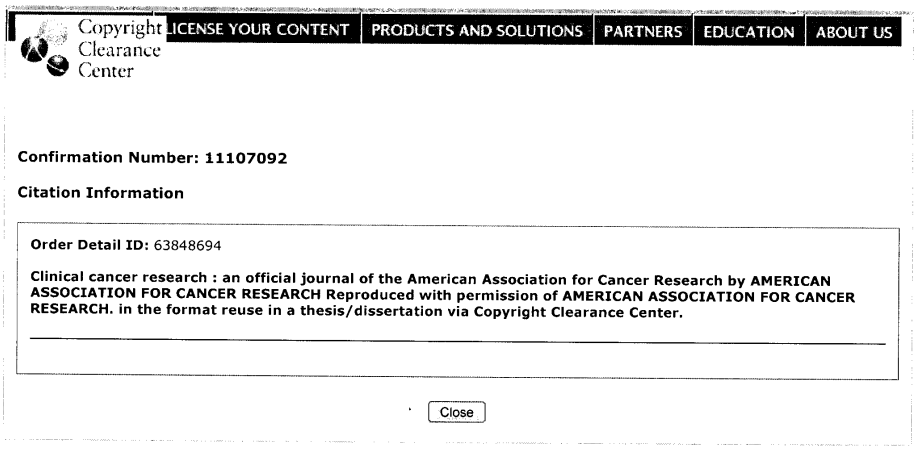

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Order detail ID:	63848876
Article Title:	Circumventing Cancer Drug Resistance in the Era of Personalized Medicine
Author(s):	Garraway, L. A. ; Janne, P. A.
DOI:	10.1158/2159-8290.CD-12-0012
Date:	Mär 01, 2012
ISSN:	2159-8290
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Volume:	2
Issue:	3
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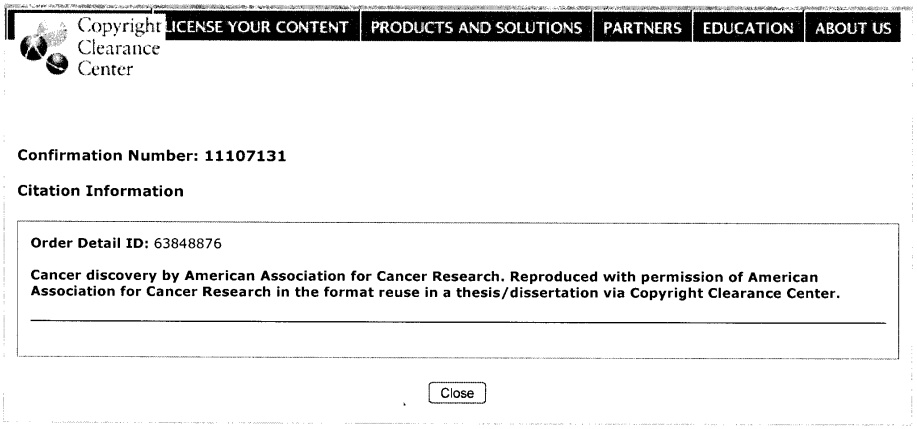

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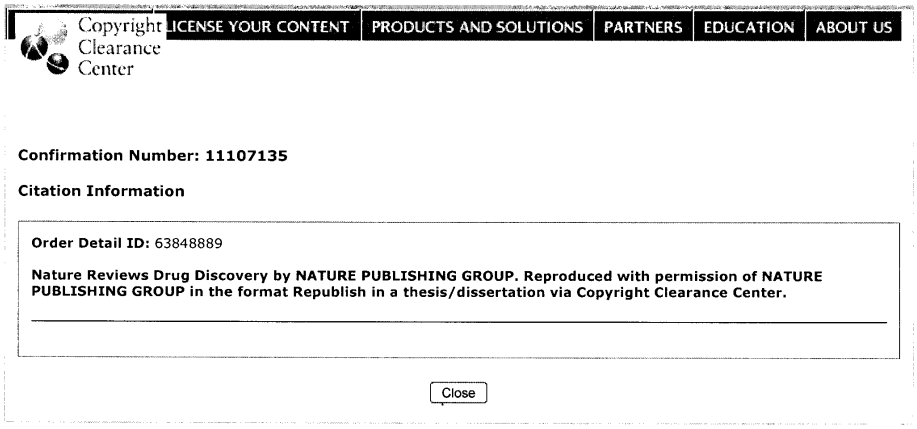
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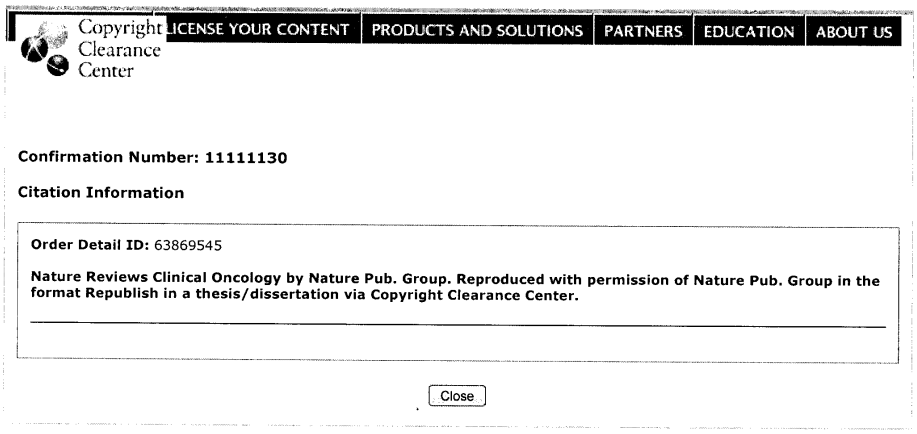

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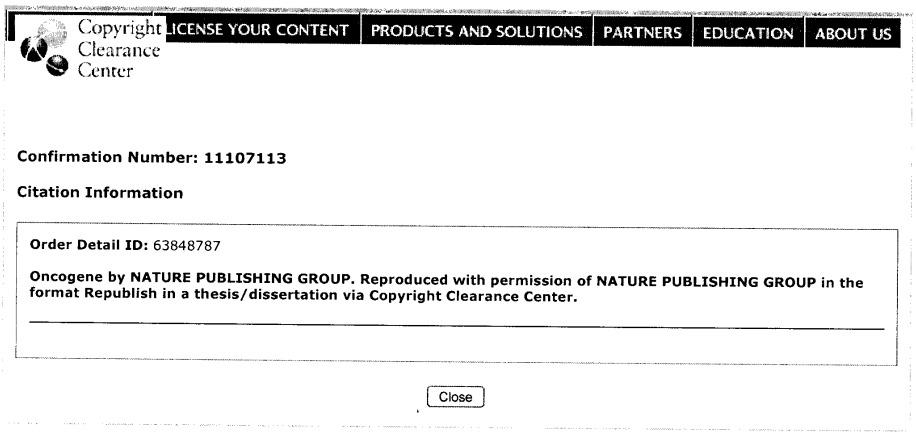

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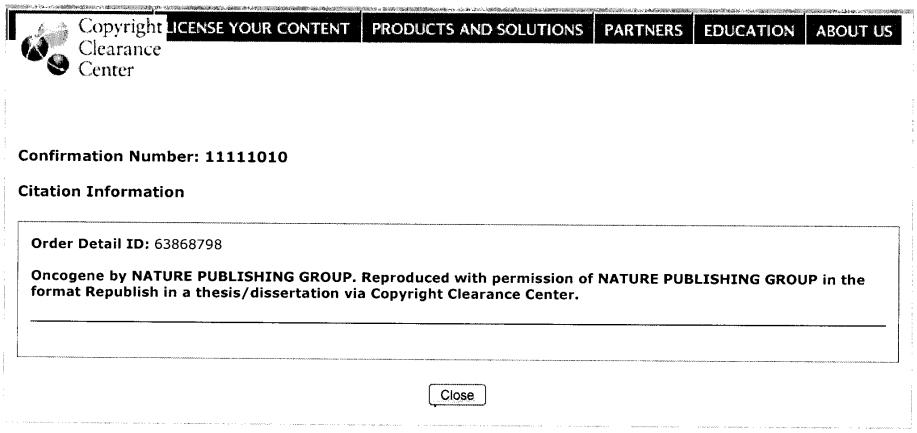

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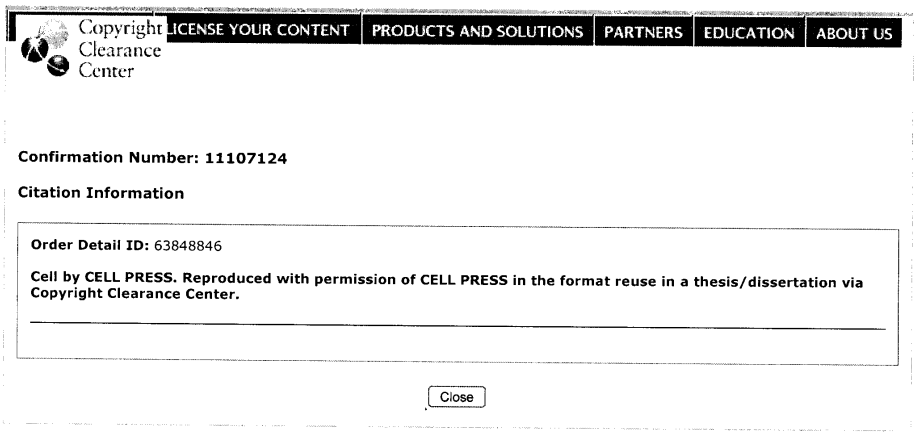

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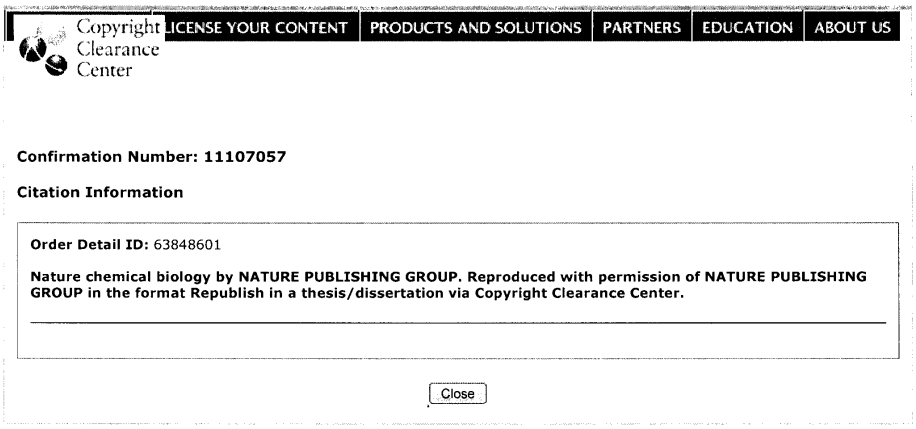
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