evotec

#RESEARCHNEVERSTOPS

Evotec’s drug discovery capabilities in: **the DNA Damage Response therapeutic area -**

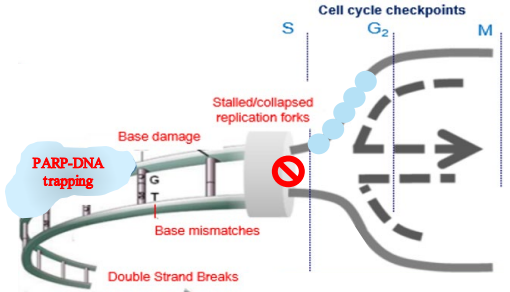
World-class models and assays

Focusing on innovative targets with first-in-class potential

Evotec oncology themes to support integrated drug discovery from concept to clinic

DNA Damage Response

Targeting the DDR and exploiting synthetic lethalities



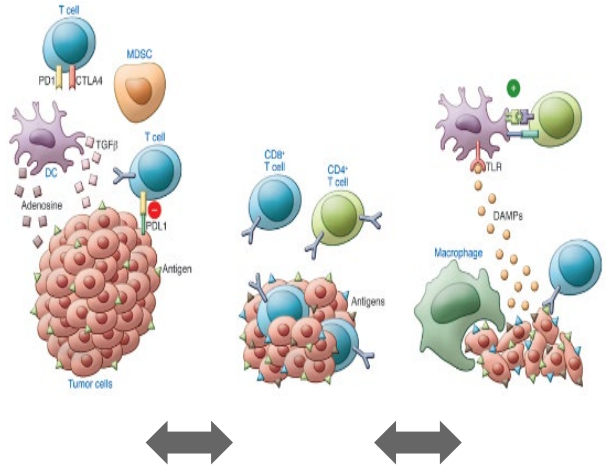
DDR Pathways:

- Base excision repair
- Single strand break repair
- Homologous recombination
- Non-homologous end-joining
- Micro-homology end-joining
- Mismatch repair
- Nucleotide excision repair
- Epigenetics
- Cell cycle checkpoints
- ALT Telomere maintenance

Single agent or combination with other targeted agents, IO and/or chemo/radiotherapy

Tumour Microenvironment

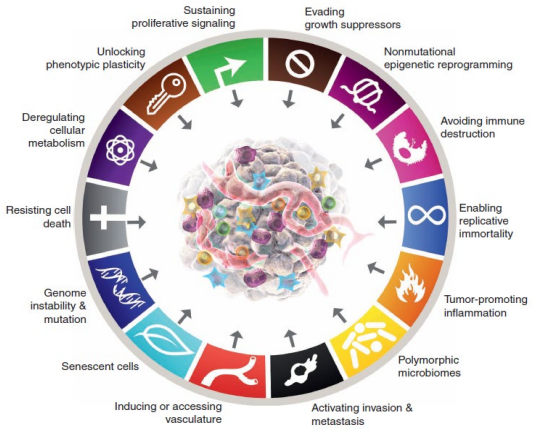
Targeting mechanisms of immune exclusion from tumours



Single agent IO agents, combinations with other targeted agents or SoC

Tumour drivers

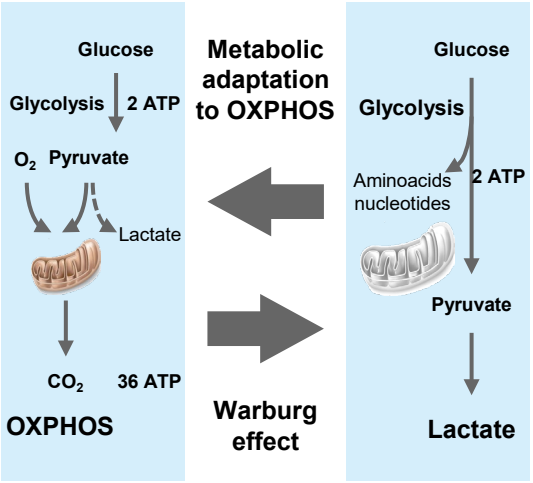
Targeting tumour drivers/oncogenes/resistance



Single agent TKIs/other targeted agents, combination with SoC

Cancer Metabolism

Targeting mechanisms of metabolic adaptation

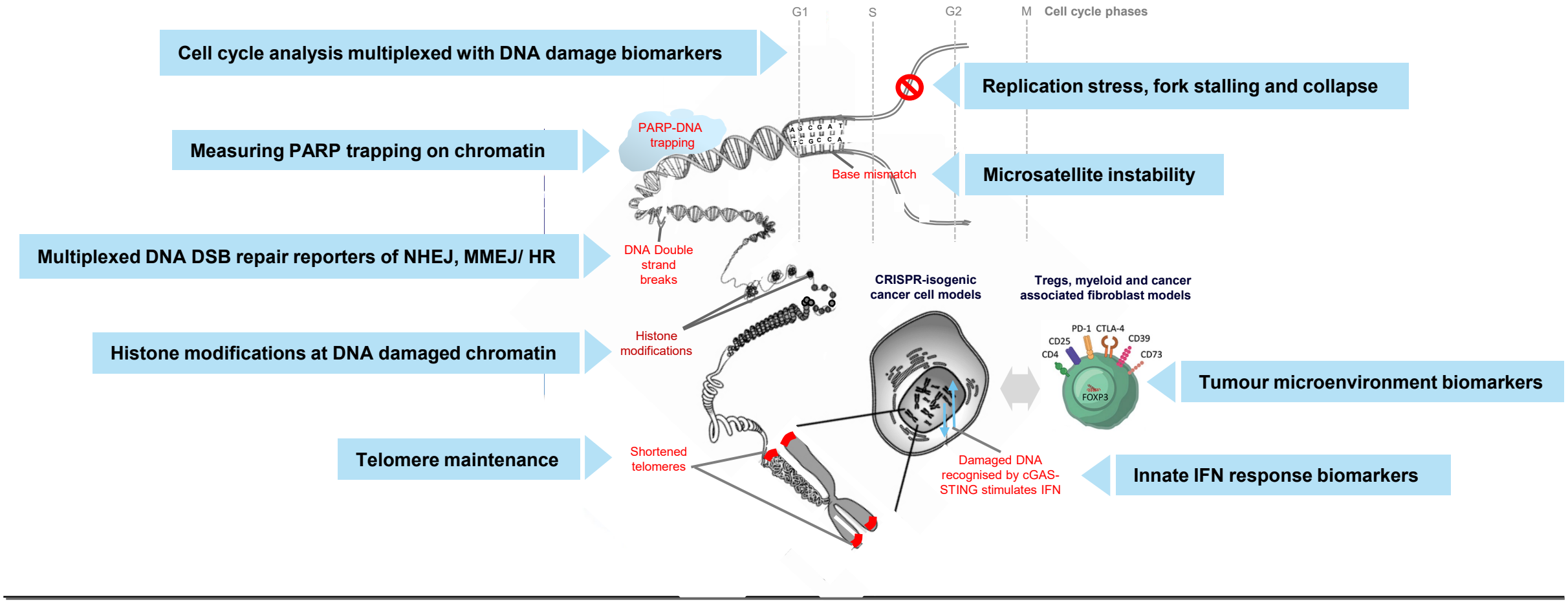


Likely combinations with SoC/targeted therapies

Contents

World-class capabilities in measuring the DNA Damage Response

Biochemical, cellular and in vivo DDR assays, biomarkers and models for drug discovery



Adapted from: REVIEW article Front. Immunol., 03 December 2019 Sec. T Cell Biology Volume 10 - 2019 | and from https://www.physio-pedia.com/Genetic_Conditions_and_Inheritance

Summary of our DDR platforms supporting drug discovery

A broad range of *in vitro* assays for screening in early drug discovery, PD biomarkers

Biochemical assays for targets of the DDR pathways:

- Parylation, kinases and ATPases (ATPase Glo), Polymerase (primer extension), Helicase (strand displacement) and Nuclease (DNA fluorescent polarisation)

Cellular assays for DDR targets and cell cycle checkpoints:

- High content analysis for RAD51 and γH2AX foci, pCHK1, pATM, pRAD50, Cell cycle, parylation. (in vivo PD biomarkers)
- Phenotypic assays - viability, proliferation (incucyte, CTG, CFA), apoptosis in synthetic lethal isogenic and resistance models :
  - HR-deficient (BRCA2-/-) vs wt (DLD1 matched pair) (grow in vivo)
  - Generating acquired PARPi- and radiation-resistant clones
- NHEJ, MMEJ, HR repair reporter assays
- PARP-trapping assay
- Replication Stress assessment using Genomic Vision (combing, fork speed etc)
- HiBit-based proteomic degradation platform (volcano plotting the expression of the proteome) – suitable for smRNAi platforms EVT offer

Control agents:

Radiation (x-ray), radiomimetics e.g.neocarzinostatin), DDRi: PARPi, ATRi, Wee1i, ATMi, topoisomerase inhibitors, carboplatin

CRISPR (whole-genome or focussed library) drop out screens

- for synthetic lethal identification, bioinformatic support and building machine-learning/AI

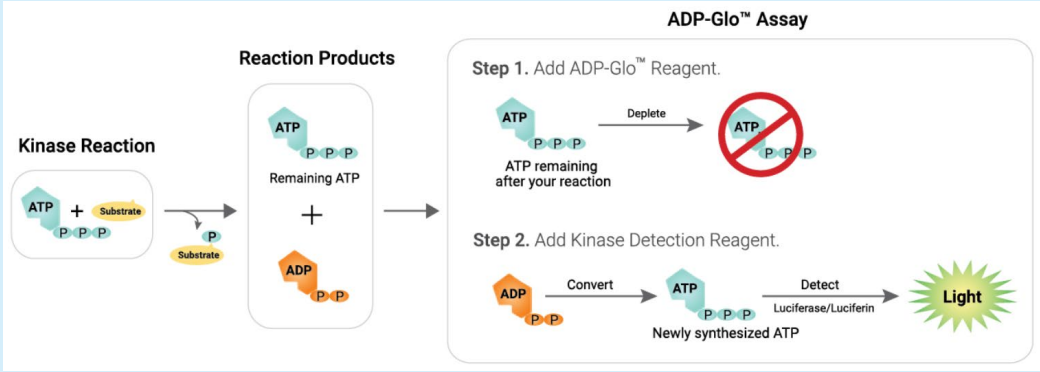
Contents

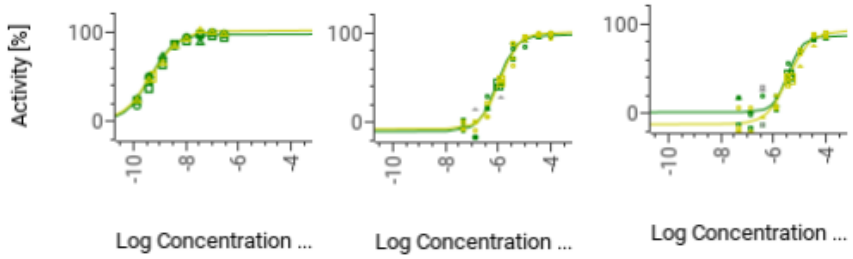
Biochemical assays: Kinase and ATPase activity

ADP-Glo™ Kinase Assay from Promega

- **Aim:**
  - Evaluate compounds potency *in vitro* for both helicases and kinases
- **Assay type:**
  - Enzymatic reaction using either truncated (kinase/ATPase domain) or full-length protein
- **Throughput:**
  - 31 compounds in dose-response - SAR driver
  - QC monitoring through assay performance (Z' and S/B) and pharmacological inhibition (reference compound IC_{50} values reproducibility)
- Assay can be developed for any kinase or helicase with ATPase activity

- **Assay principle:**
  - Measurement of ATP conversion using the Promega ADP-Glo™ Kinase assay. Upon ATPase/kinase activity, robust and stable luminescent signal is generated



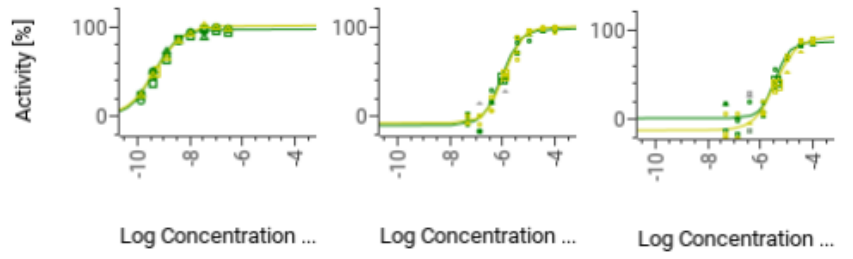


	Compound X	Compound Y	Compound Z
pIC50	9.3	5.9	5.4

Biochemical assays: Polymerase activity

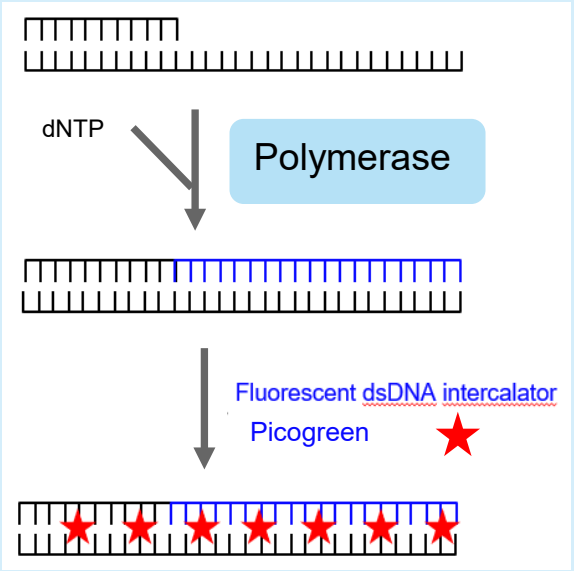
Primer extension assay

- **Aim:**
  - Evaluate compounds potency *in vitro* for polymerases
- **Assay type:**
  - Enzymatic reaction using either truncated (polymerase domain) or full-length protein
- **Throughput:**
  - 31 compounds in dose-response – SAR driver
  - QC monitoring through assay performance (Z' and S/B) and pharmacological inhibition (reference compound IC_{50} values reproducibility)
- Assay can be developed for any polymerase



	Compound X	Compound Y	Compound Z
pIC50	9.3	5.9	5.4

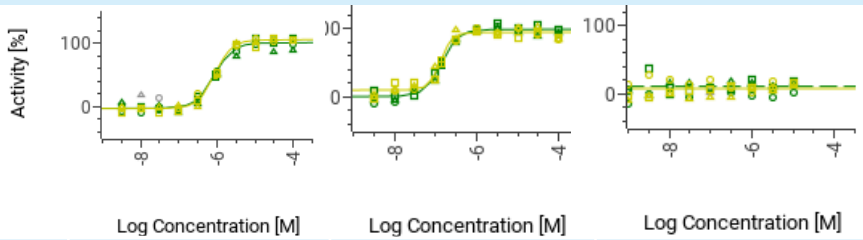
- **Assay principle:**
  - Quantification of dsDNA synthesis upon polymerase activity using the fluorescent and dsDNA-specific dye Picogreen®



Biochemical assays: Polymerase activity

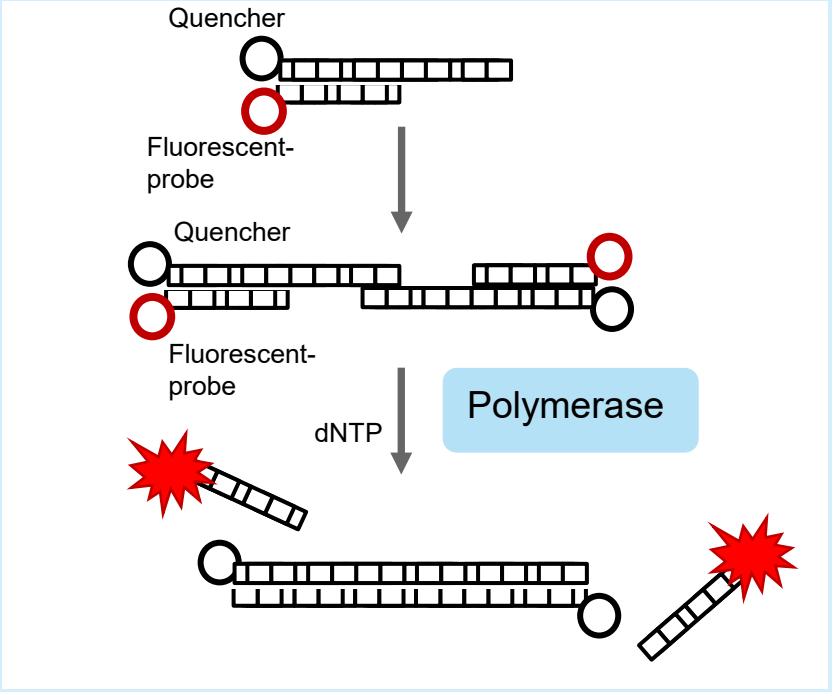
Strand-displacement assay

- **Aim:**
  - Evaluate compounds potency *in vitro* for polymerases
- **Assay type:**
  - Enzymatic reaction using either truncated (polymerase domain) or full-length protein
- **Throughput:**
  - 31 compounds in dose-response – SAR driver
  - QC monitoring through assay performance (Z' and S/B) and pharmacological inhibition (reference compound IC_{50} values reproducibility)
- Assay can be developed for any polymerase and can be considered as orthogonal validation method



	Compound X	Compound Y	Compound Z
pIC50	6.0	6.8	>4

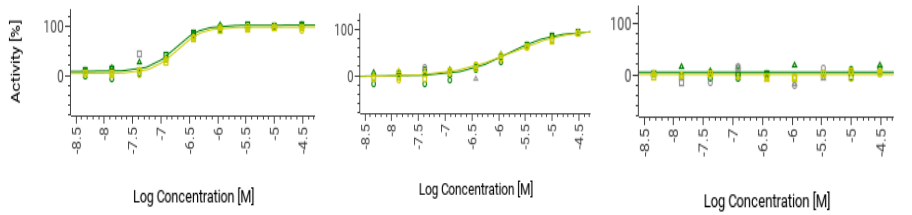
- **Assay principle:**
  - Quantification of polymerase activity relying on the ability of the polymerase to displace downstream DNA encountered during synthesis. This displacement prevents the quenching of the fluorescent probe



Biochemical assays: Helicase activity

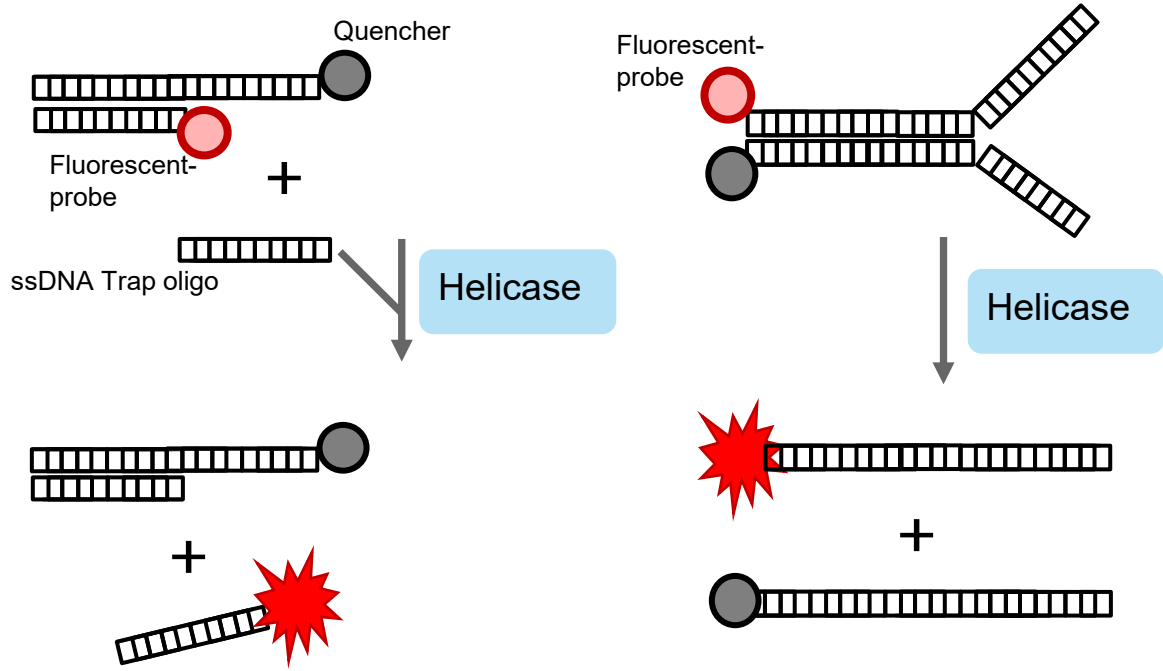
Strand-displacement assay

- **Aim:**
  - Evaluate compounds potency *in vitro* for helicases
- **Assay type:**
  - Enzymatic reaction using truncated protein (helicase domain)
- **Throughput:**
  - 31 compounds in dose-response - SAR driver
  - QC monitoring through assay performance (Z' and S/B) and pharmacological inhibition (reference compound IC_{50} values reproducibility)
- Assay can be developed for any helicase displaying DNA-unwinding activity



	Compound X	Compound Y	Compound Z
pIC50	6.7	5.8	>4.5

- **Assay principle:**
  - Fluorometric measurement of ssDNA substrate arising from unwinding activity of the helicase. The DNA displacement prevents the quenching of the fluorescent probe



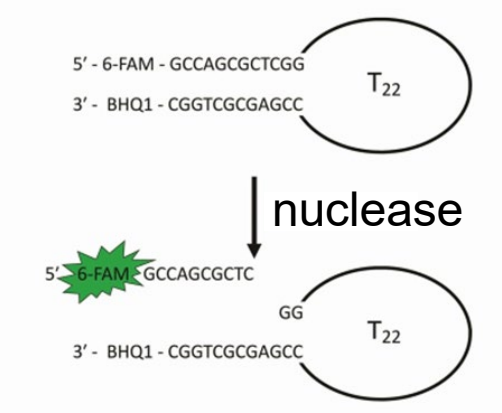
  - Assay can be developed either with or without ssDNA trap to stabilise the fluorescent signal

Biochemical assays: Nuclease activity

Choice of DNA quenching or protein-protein interaction determination if in an active complex

Enzymatic assay (fluorescent DNA substrate)

- E.g. for endonuclease actviity
- Synthesis of Molecular beacon probes
  - Reflexion on another fluorochrome to avoid compounds autofluorescencing

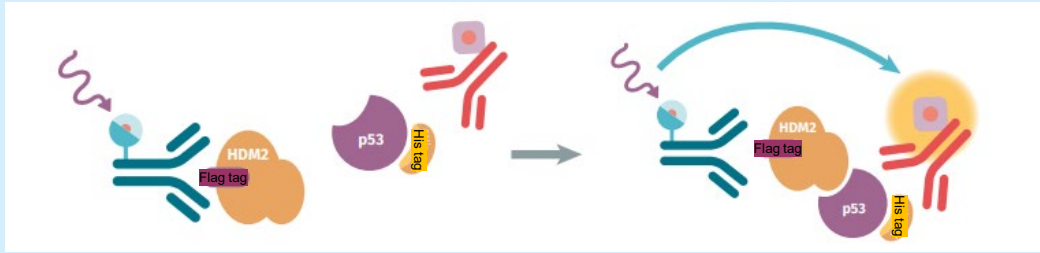


Dye	Quencher
6-FAM	BHQ1
HEX	Lowa Black FQ
TET	Lowa Black FQ
TYE 563 / TYE 665	Lowa Black RQ

Bowles M. et al., Nucleic Acids Research, 2012, Vol. 40, No. 13

PPI assay (HTRF assay)

- Working on 2 independent proteins
- (HTRF technology
  - MAB Anti 6His / Mab Anti Flag
  - Choice of acceptor and donor on each MAB (d2, Eu-cryptate, Tb cryptate, XL665). E.g. as for p53-MDM2



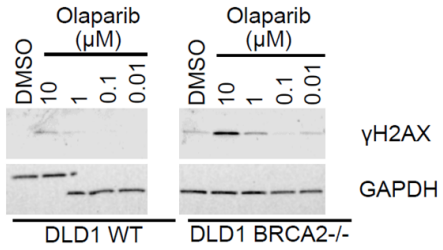
- A counter-assay based on a doubled-tagged peptide to filter out positive compounds interfering with the readout (Kit interference available).

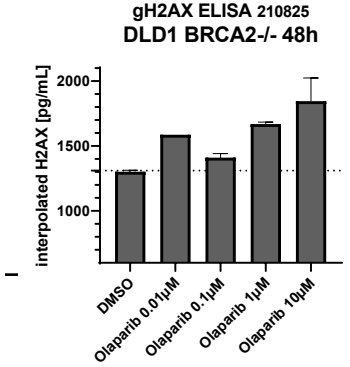
Contents

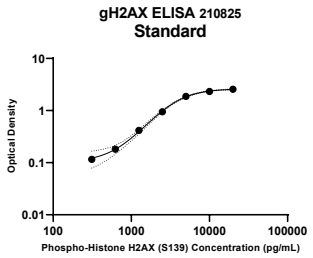
Cellular biomarkers of DNA Double Strand Break detection

Quantify γH2AX foci and Rad51 as a proxy for DSB induction using high-content imaging

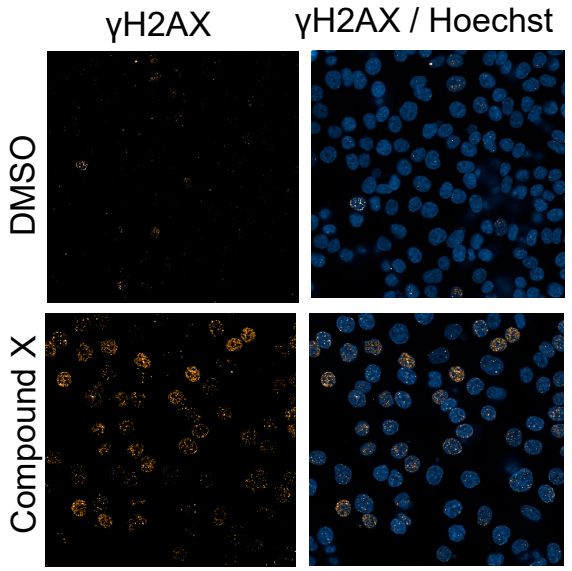
- **Rationale:**
  - Double-Strand breaks induces γH2AX foci formation.
  - Phosphorylation on Serine 139 is mediated by the kinases ATM, ATR and DNA-PK and is an early cellular response to DSBs
- **Throughput:**
  - 27 compounds in dose-response
  - Suitable for Tier1 assay
- **Possibility of multiplexing** (Up to 4 colours): with other biomarkers (ex Rad51 for HR) or with cell cycle marker (ex EdU, H3S10-P)
- Alternative technologies to be considered for *in vivo* studies:
  - Detection of γH2AX by Western Blot and ELISA

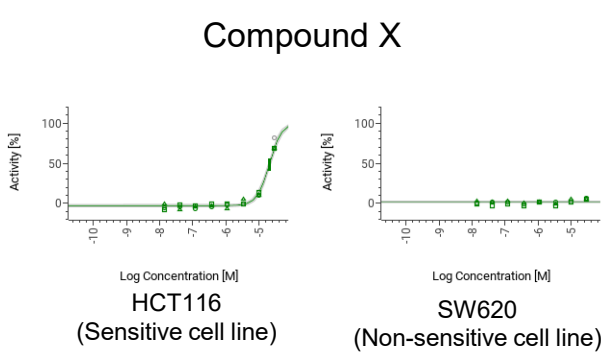






Case study: Cpds screening in HCT116 using Operetta® technology





Activity expressed as % of γH2AX-positive cells
-Etoposide used as 100% activity

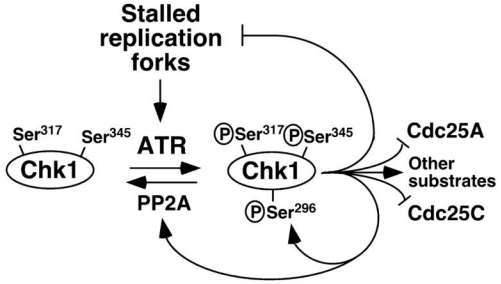
Readout	Cell line	pIC50
% gH2Ax positive cells	HCT116	4.67
% gH2Ax positive cells	SW620	inactive

γH2AX detection also developped in SW480, RKO, DLD1, Kuramochi, U2OS cell lines

Cellular assays: Phosphorylation events during DDR signalling

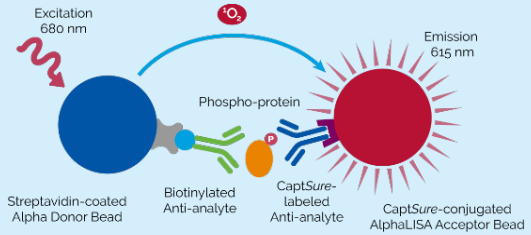
Phosphorylation of Chk1 - AlphaLISA® SureFire® Ultra™ p-CHK1 (Ser345)

- **Rationale:**
  - Direct monitoring of Chk1 phosphorylation
  - Indirect monitoring of Replication stress: Replication stress induces phosphorylation of checkpoint kinases Chk1 through ATR activation

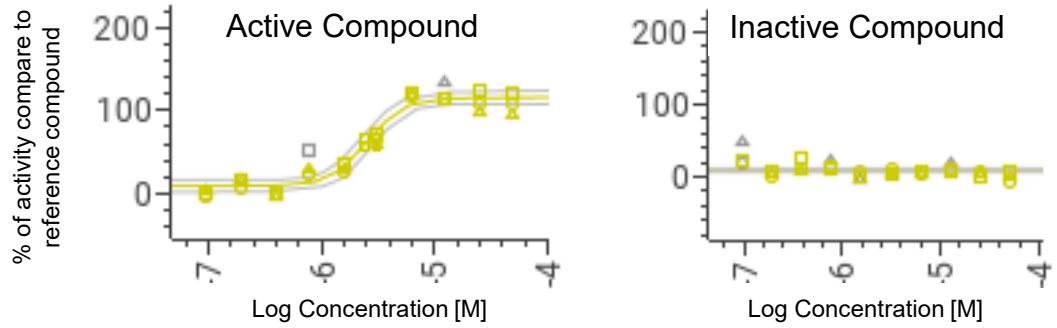


- **Throughput:**
  - 27 compounds in dose-response
  - Suitable for Tier1 assay
- **Additional targets for potential assay development using AlphaLISA:**
  - p-ATM (S1981) for DSB signalling as alternative to γH2AX
  - p-Chk2
  - p-RAD50
  - p-KAP1

- **Assay principle:** The AlphaLISA® SureFire® Ultra™ p-Chk1 (Ser345) assay is a sandwich immunoassay for quantitative detection of phospho-Chk1 (Ser345) in cellular lysates using Alpha Technology



Case Study: Phosphorylation of Chk1after treatment (21h) in dose-response of inhibitor in Colo-320DM cells



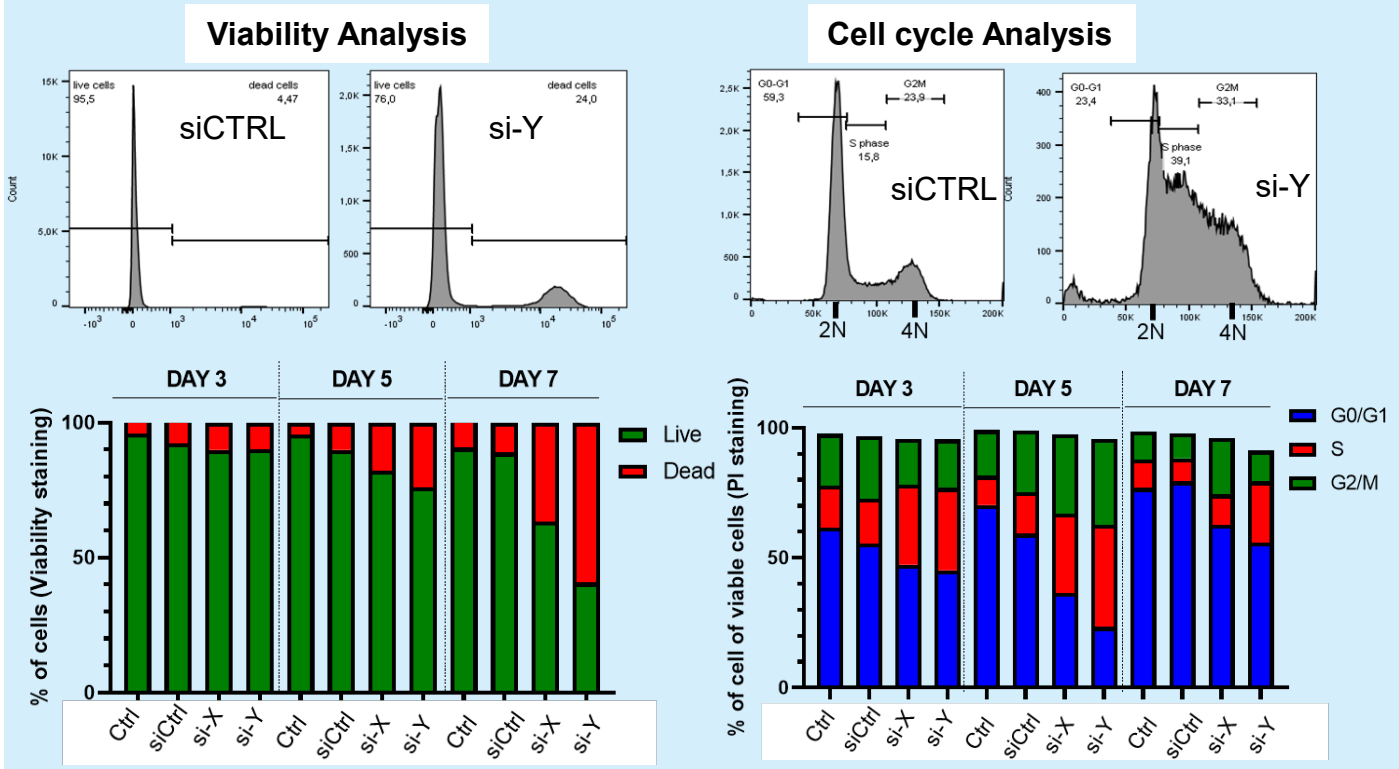
Inhibition of protein X induces replication stress and subsequent phosphorylation of Chk1

Cellular assays: Flow cytometry

Viability and cell cycle analysis by FACS

- **Rationale:** DNA damage induces cell cycle arrest and/or cell death
- **Assay principle:** Analysis of cell viability (Via far Red Fluorescent dye) and cell cycle (Propidium iodide)
- **FACS park :**
  - BD FACS Canto II
  - BD Fortessa X20
  - Biorad ZE5
- **Throughput:**
  - Not suitable for Tier1
  - More suitable for target validation or Tier2 or Tier3 assays
- Possibility of co-staining with cell cycle marker (ex. Edu, H3S10-P) and/or with DNA damage marker (ex. γH2AX, pKAP1*)

Case study: Target validation FACS analysis of BT20 cells transfected with siRNA



Depletion of the target induces cell death and accumulation in S phase

*not yet developped

Cellular assays: Replication stress markers

High content microscopy to count 53BP1 bodies and micronuclei

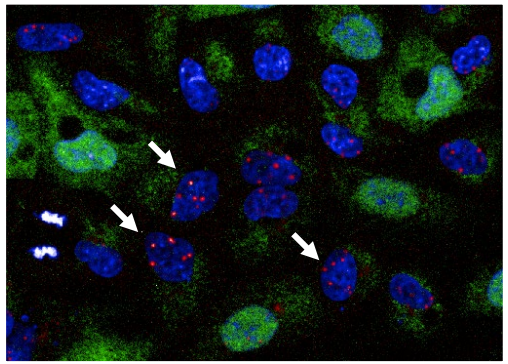
- **Rationale:** Replication stress increases chromosomal lesion transmitted to daughter cells, characterised by cells in G1 with 53BP1 bodies and micronuclei
- **Assay principle:** Quantification of 53BP1 bodies and micronuclei in G1-cells using high-content imaging
- **Throughput:** up to 27 compounds in dose-response
- **Assay to be adapted for the cell line of interest for optimal condition :**
  - Cell density
  - Kinetic of inhibitor exposure
  - DMSO tolerance
  - 96/384 well plate format

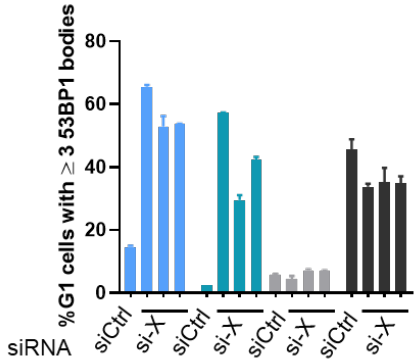
Operetta® technology

- Possibility to multiplex with Rad51 foci
- **For 2023 we will be purchasing the Genomic Vision and mastering quantification of replication fork speed**

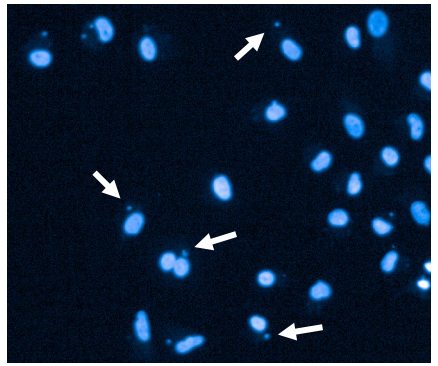
Case study: Micronuclei count under siRNA treatment using Operetta® technology

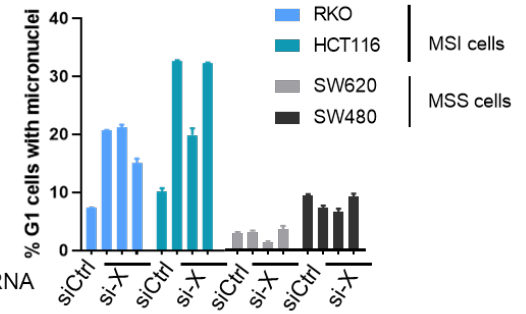
53BP1 bodies





Micronulei





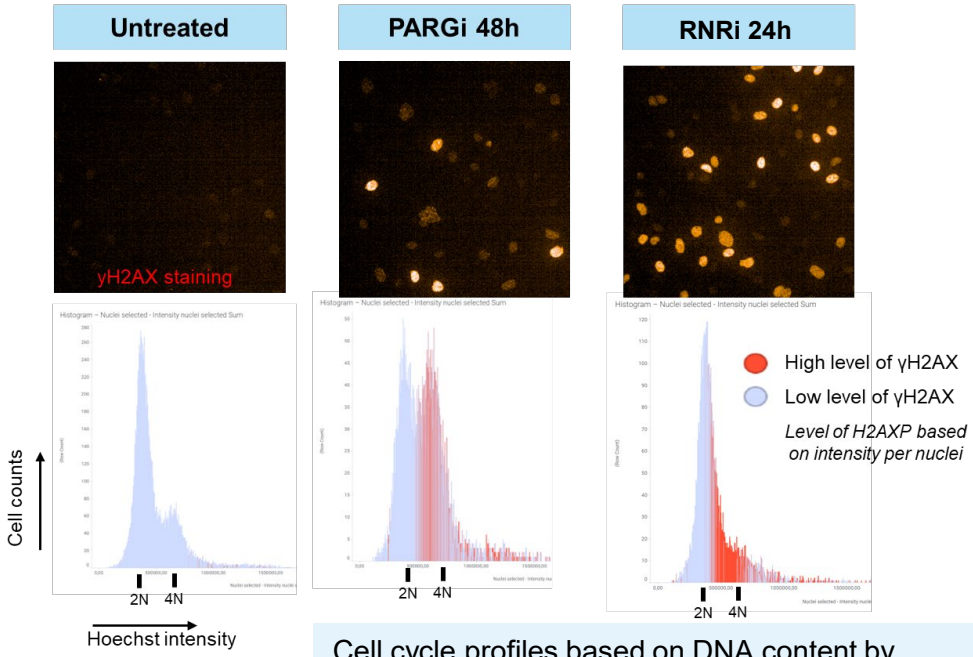
Downregulation of X by siRNA increases 53BP1 bodies and micronuclei specifically in MSI cells

Evaluation of Replication stress markers

H2AX-P in S phase population ; RPA-P and KAP1-p in response to replicative stress

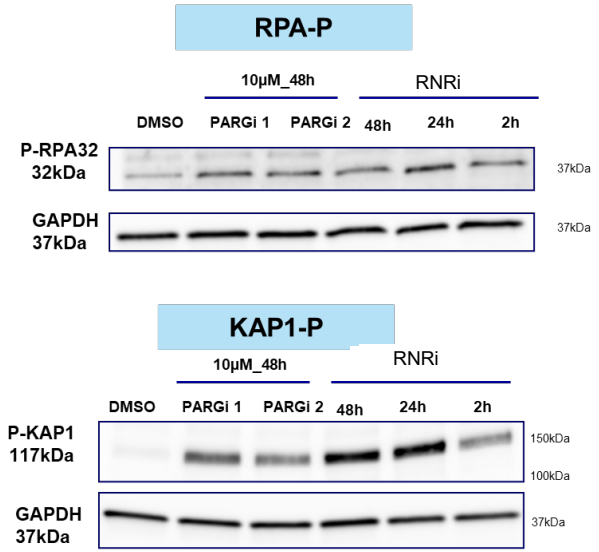
- **Rationale:** Replication stress induces DSBs in S phase population and induces a change on cell cycle profile.
- **Assay principle:**
  - Cell cycle profile base on DNA content quantified with high throughput imaging : 2N peak corresponds to G1 phase & 4N peak correspond to G2 and Mitotic cells
  - Quantification of H2AX-P intensity level in nuclei upon drug treatment
  - Overlay of relative H2AX-P signal with cell cycle profile : if DNA damage is induced in S phase → replication stress
- **Replication stress / DDR marker by western blot :**
  - pRPA (s4-8) antibody
  - pKAP1(s824)

Induction of Replication stress in kuramochi cells



Cell cycle profiles based on DNA content by high throughput imaging ; colour code: blue corresponds to undamaged nuclei and dark red to high level of H2AX-P

Western-Blot of replication stress markers



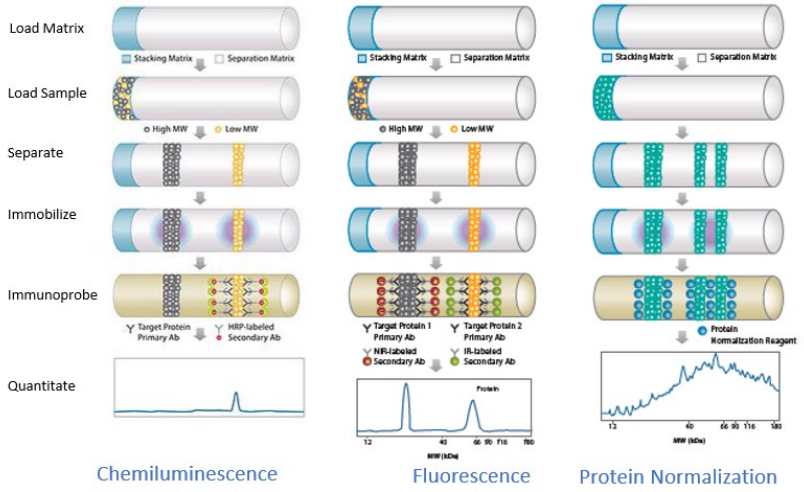
48h of PARGi in kuramochi cell line induces replication stress : S phase and G2/M cell are associated with DSBs. RNRi induces a depletion of the dNTP pool and a subsequent replicative stress (G1 arrest and DNA breaks in S phase)

Simply-western blot / JESS technology to assess DDR

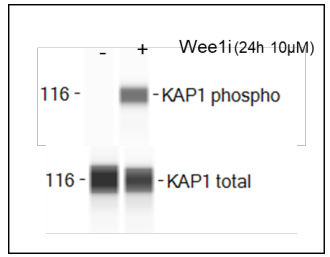
Quantitative technology to study DDR on protein level

- **Rational :** the DDR is mediated by post-traductional modifications such as phosphorylation, ubiquitylation and ADPr, these regulations can be assessed at the protein level by western blot or with the JESS technology
- **Simply-western blot / JESS technology**
  - Automated Western Blotting using capillaries, 2kDa to 440kDa, 3-4 logs dynamic range
  - Use of chemiluminescence or fluorescence (2 lasers)
  - 24 samples per run (up to 96 well plate per day)
  - Normalization :internal or total protein loaded (3rd dye)
- **DDR Antibody validated with the JESS in EVT**
  - H2AX-P
  - ADPr
  - KAP1/P
  - KAP1 total
- **Case study:** KAP1 is a target of ATM kinase in response to DNA damage and is induced upon treatment with Wee inhibitor (inducing replication stress)

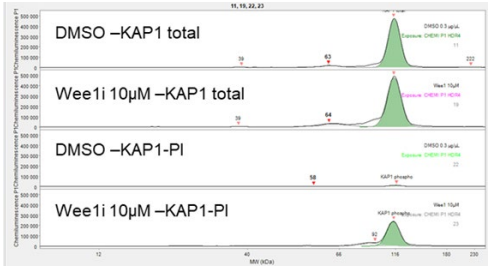
The Simple Western Technology



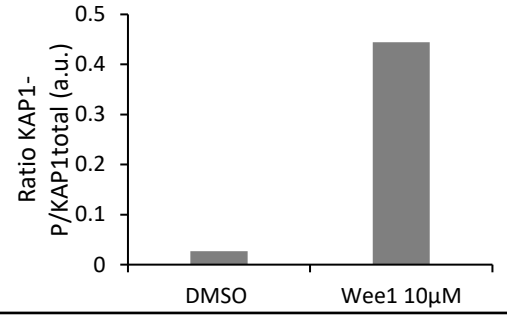
Experiment design ;
Protein lysate of cancer cells treated with DMSO or Wee1i for 24h (inducing replication stress)
Analysis by JESS: KAP1 total and KAP1-P validated in JESS (linearity and saturation)
Quantification : Ratio analysis: KAP1-P/KAP1total



Peak detection



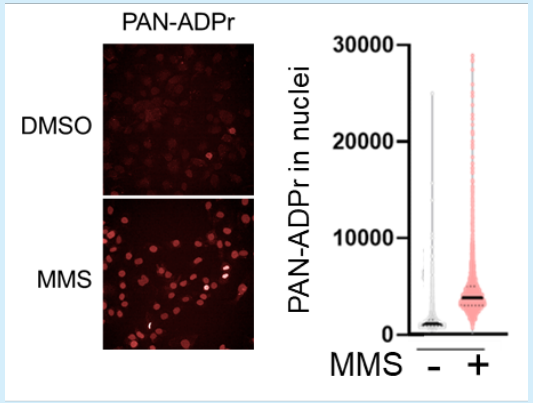
Quantification



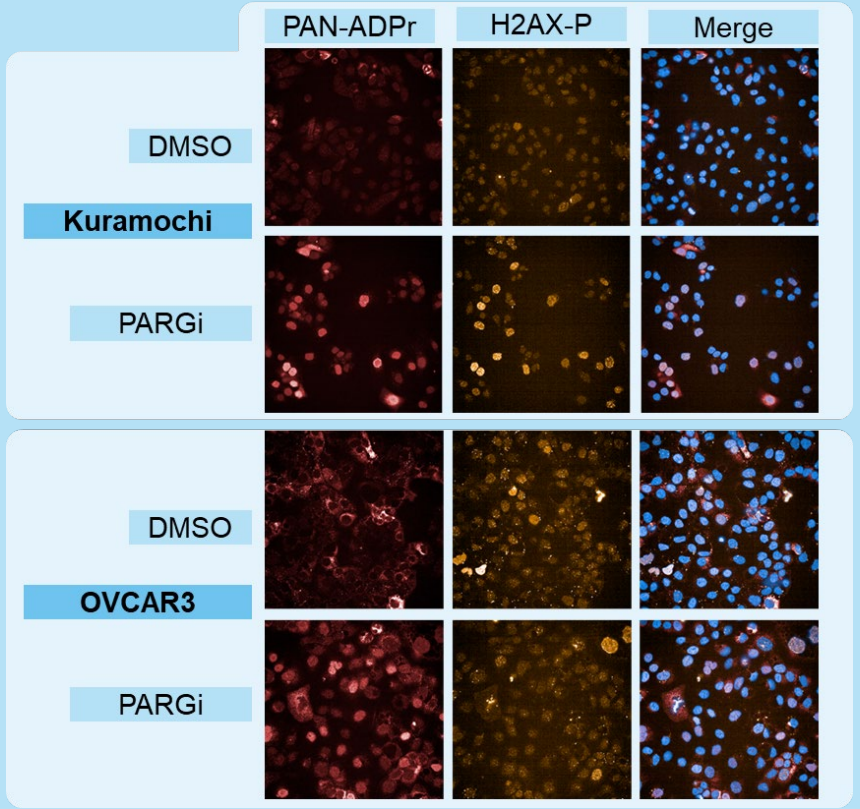
ADPr staining analysis in cells

Quantifying of PAN-ADPr signal using high-content imaging

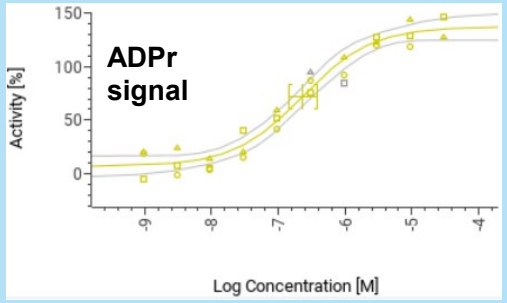
- **Rationale:** In response to DNA damage (SSBs, Replication colapse, DSBs), PARPs family enzymes induce ADP-ribosylation (poly or mono ADPr) of target proteins
- **Assay principle:**
  - Fixation and Staining have been optimised in order to quantify nuclei PAN-ADPr
  - ADPr (biomarkers of single Strand Break) can be multiplexed with H2AX-P (DSBs marker)
  - Throughput:
    - 27 compounds in dose-response
    - Suitable for Tier1 assay
- Global PAN-ADP ribosylation in response to MMS treatment & quantification of individual nuclei

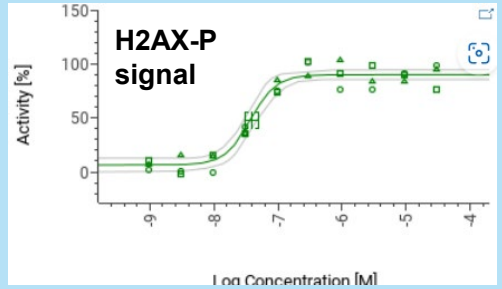


- **Case study:** PARGi has been described (Pillay et al 2019) to induces ADPr signal in all cell lines, but to induces DSBs only in specific cell lines (ex.Kuramcohi)



Cpds screening & profiling using Operetta® technology (in Kuramochi)





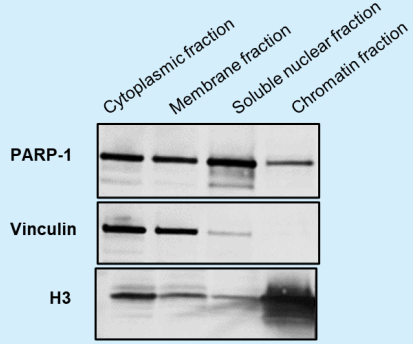
PARP1 chromatin localization / trapping

Western blot and IF approaches to look at chromatin binding protein

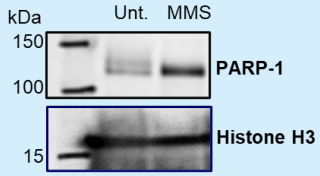
- **Rational :**
  - PARP1 protein has a high affinity for DNA break ends (single and DSBs) and is recruited at the chromatin in response to DNA damage upon treamtnt
  - PARP inhibitors, in addition to the inhibition of enzymatic activity, traps the PARP protein at the chromatin. PARP trapping causes replication stress and, in HR deficient cell cline, cell death
- **Western blot approach :**
  - Cell are fractionated fllowing standardised processes and PARP level in the chromatin fraction is analysed upon drug treatment
  - Reference method for PARP trapping , but low throuput
- **IF approach:**
  - Pre-extration with CSK buffer to remove unbound PARP protein.
  - Fixation and anti-PARP IF
  - HTS / 384 well plate format

Western blot approach :

Chromatin extraction protocol:



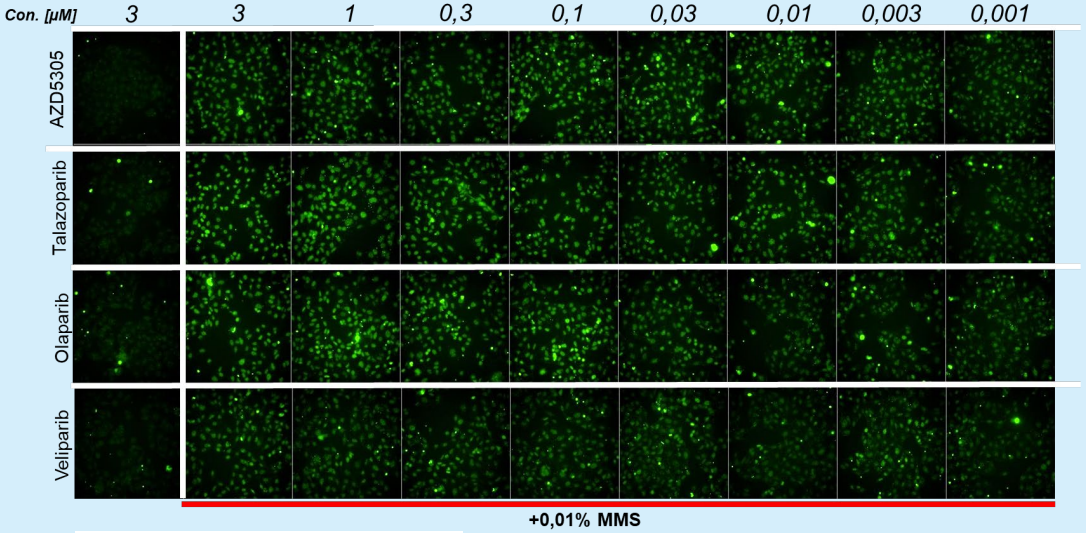
PARP1 level in the chromatin fraction in response to MMS

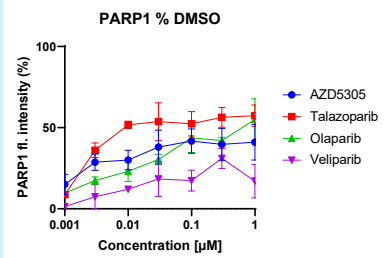


- *PARP1 relocalisation on chromatin upon MMS treatment*

Immunofluorescence approach :

PARP1 trapping upon PARGi treatment + MMS :
Fixation procedure = nuclei isolation extraction: CSK pre-extraction buffer prior to PFA fixation





- *Increase of PARP1/PARP2 staining in PARPi+MMS DLD1 wt treated cells*
- *Intensity of PARP signal positively correlates with the dose of PARPi*
- *Veliparib shows the lowest capability of PARP trapping, while PARP staining is highest in condition of treatment with strong trapper Talazoparib.*

CSK Cytoskeleton buffer

Assaying DNA double strand break repair

The four major pathways to repair DNA Double-Strand Breaks (DSBs)

The four major pathways to repair DNA Double-Strand Breaks (DSBs)

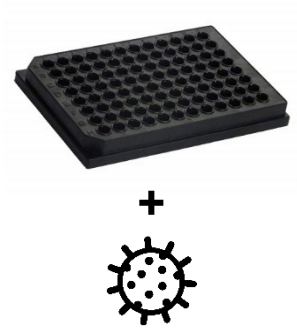
- Unprocessed DSBs can be repaired through classic **non-homologous end joining (cNHEJ)** allowing the two ends of the DSB to be re-ligated
- DSB ends can also be processed by the MRN complex and its interacting factors to yield short 3’ ssDNA overhangs. The short 3’ ssDNA overhangs can then be channelled into the **microhomology-mediated end joining (MMEJ) pathway**
- Alternatively, the DSB ends can undergo further long-range resection by either EXO1 or BLM/DNA2. These longer ssDNA overhangs are first bound by RPA and can then be channelled into the **Single Strand Annealing (SSA) pathway**, which is mediated by the protein RAD52.
- Alternatively, the RPA-ssDNA can serve as a substrate for the RAD51 filament assembly, allowing the resulting DNA intermediates to be directed towards repair by **Homologous Recombination (HR)**. For HR, both ssDNA and dsDNA templated homology repair (HDR) pathways are used

https://pubmed.ncbi.nlm.nih.gov/33896583

GFP report assay: Monitor DSB repair by NHEJ and MMEJ pathways

Workflow for compound screening

Cell seeding → Induction of DSBs/compound treatment → Quantification of GFP+ cells → Data analysis/results



Cells (stable integration):

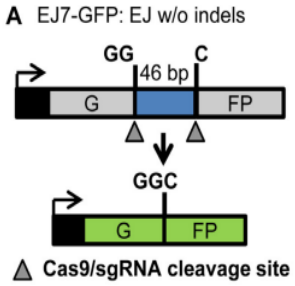
GFP report cassette for:

- NHEJ repair
- MMEJ repair

Lentiviral particles for DSB:

Cas9_WT/sgRNA/mCherry

Non-homologous end joining **(NHEJ)**

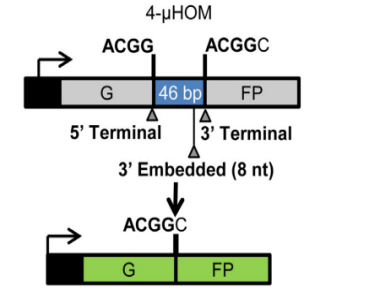


Positive control inhibition

DNAPKi (AZD7648)

Ku70/80 siRNA

Micro-mediated end joining **(MMEJ)**



4-µHOM Terminal = 5' Terminal + 3' Terminal

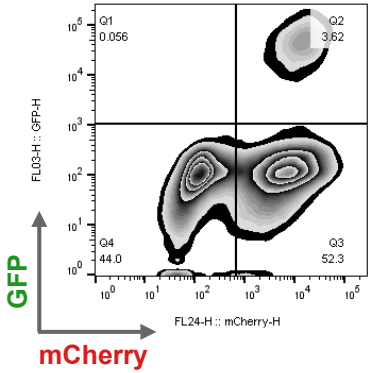
4-µHOM Embed = 5' Terminal + 3' Embedded

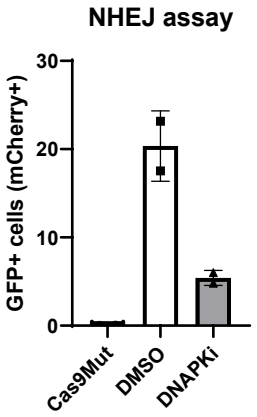
Positive control inhibition

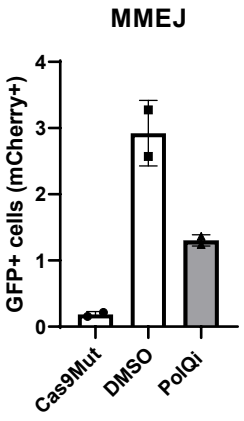
PolQi (ART812)

FEN1 siRNA

Flow cytometry







Bhargava et al., 2018. C-NHEJ without indels is robust and requires synergistic function of distinct XLF domains. Nature Communications. DOI: 10.1038/s41467-018-04867-5

evotec

Cas9

GFP report assay: Monitor DSB repair by HR and SA pathways

Workflow for compound screening

Cell seeding → Induction of DSBs/compound treatment → Quantification of GFP+ cells → Data analysis/results

+

Cells (stable integration):

GFP report cassette for:

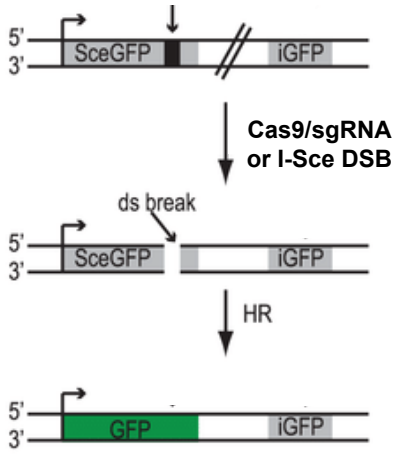
- HR repair
- SSA repair

Lentiviral particles for DSB:

Cas9_WT/sgRNA/mCherry

I-Sce/mCherry

Homologous Recombination **(HR)**

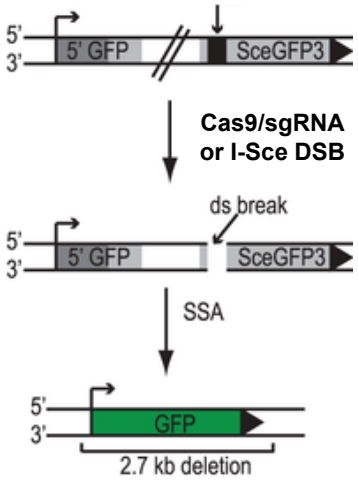


Positive control inhibition

ATMi (AZD1390)

Rad51 siRNA

Single Strand Annealing **(SSA)**



Positive control inhibition

Rad52i (D-I03)

Rad52 siRNA

Flow cytometry

Assay under development

Reference: Bhargava et al., 2018. C-NHEJ without indels is robust and requires synergistic function of distinct XLF domains. Nature Communications. DOI: 10.1038/s41467-018-04867-5

Traffic Light reporter assays for HR and NHEJ pathways

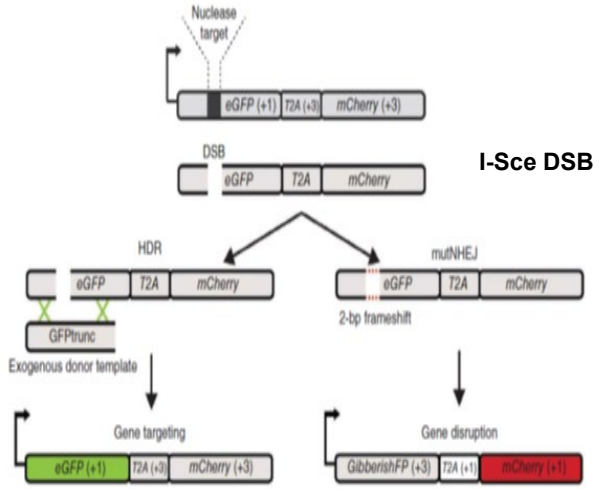
Workflow for compound screening

Cell seeding → Induction of DSBs/compound treatment → Quantification of GFP+ cells → Data analysis/results

+

Cells (stable integration):
GFP/mCherry report cassette for both HR and NHEJ repair

Lentiviral particles for DSB:
I-Sce



I-Sce DSB

Positive control (HR)
ATMi (AZD1390)
ATM-siRNA

Positive control (NHEJ)
DNAPKi (AZD7648)
Ku70/80 siRNA

Flow cytometry

Assay under development

Reference: Bhargava et al., 2018. C-NHEJ without indels is robust and requires synergistic function of distinct XLF domains. Nature Communications. DOI: 10.1038/s41467-018-04867-5

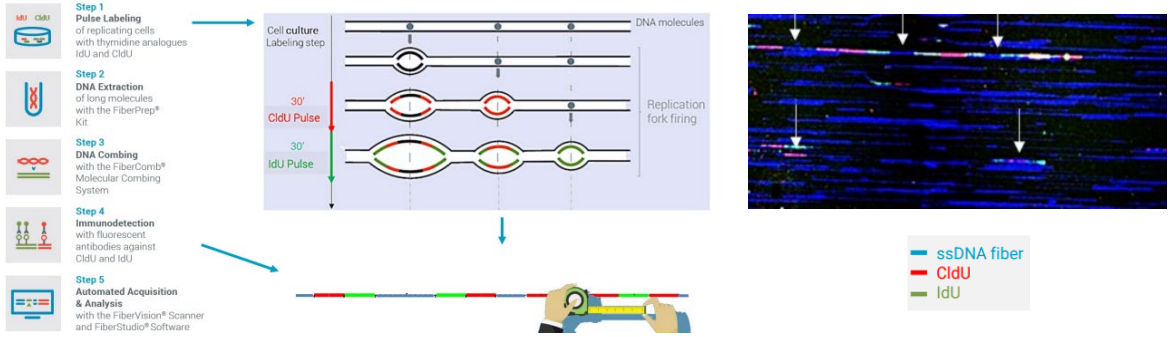
Molecular Combing Platform - Genomic Vision®

Evotec gaining access to Genomic Vision® technology

- **Rationale:** Replication stress is one of the hallmarks of cancer cells and is an attractive target for anti-cancer molecules. The DNA combing is a cutting-edge technology that allows the analysis of individual stretched DNA molecules
- **DNA combing plateform:** includes the Molecular Combing System to stretch the DNA, the automated scanner and the Molecular Combing Analysis software
- **Replication combing assay:**
  - Consists to pulse label the DNA with nucleotide analogue (IdU CldU) to monitor DNA replication and replication stress
  - After labelling, DNA extraction, stretching, immunostaining, and image acquisition, computing analysis measures:
    - Replication speed (fork velocity, fork progression/speed),
    - Initiation rates / Replication firing (number of activated origins / total length of DNA molecule)
    - Fork symmetry (length of inter-origin distance).
- **Throughput:** limited throughput, suitable for target validation, and selected compounds characterization

- Genomic Vision® platform & composition :

- Replication combing assay



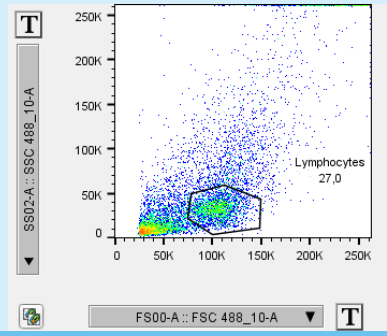
Flow Cytometry on whole blood - ADP-ribosylation signal

ADPr-of protein (PAR) as a biomarker in human blood

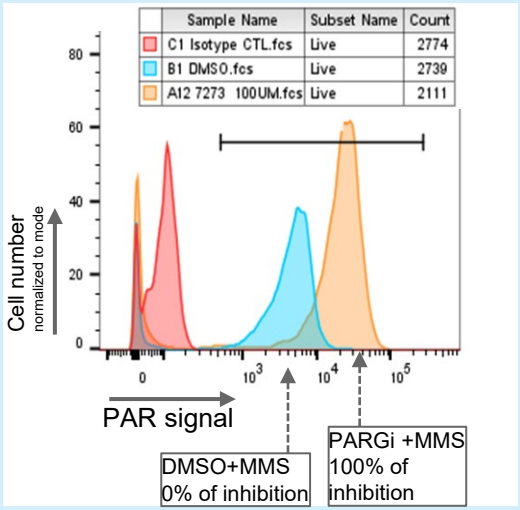
- **Rationale:** Using human whole blood from healthy donors to look at DDR biomarker and target engagement
- **Assay principle:**
  - Collection of fresh whole blood from donors
  - Lysis of red blood cells
  - Treatment in DR
  - Incubation time
  - Fixation, staining with anti-ADPr/PAR antibody
  - FACS acquisition
  - DR analysis
- **Throughput:**
  - 3 donors a week
  - 3 compounds in DR / per 96 well plate; one plate /donor/week
  - Suitable for Tier2 assay

Case study: Human Whole blood were collected, lysed, treated with PARGi for 48h, and ADPr challenged with a short MMS treatment. After fixation, immunostaining and FACS acquisition, ADPr signal induced in a DR were analysed

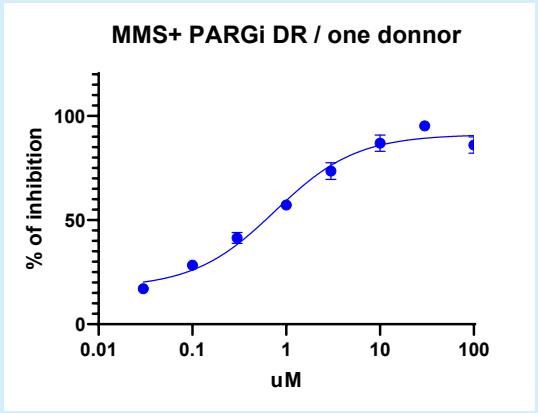
Gating strategy:



PAR staining



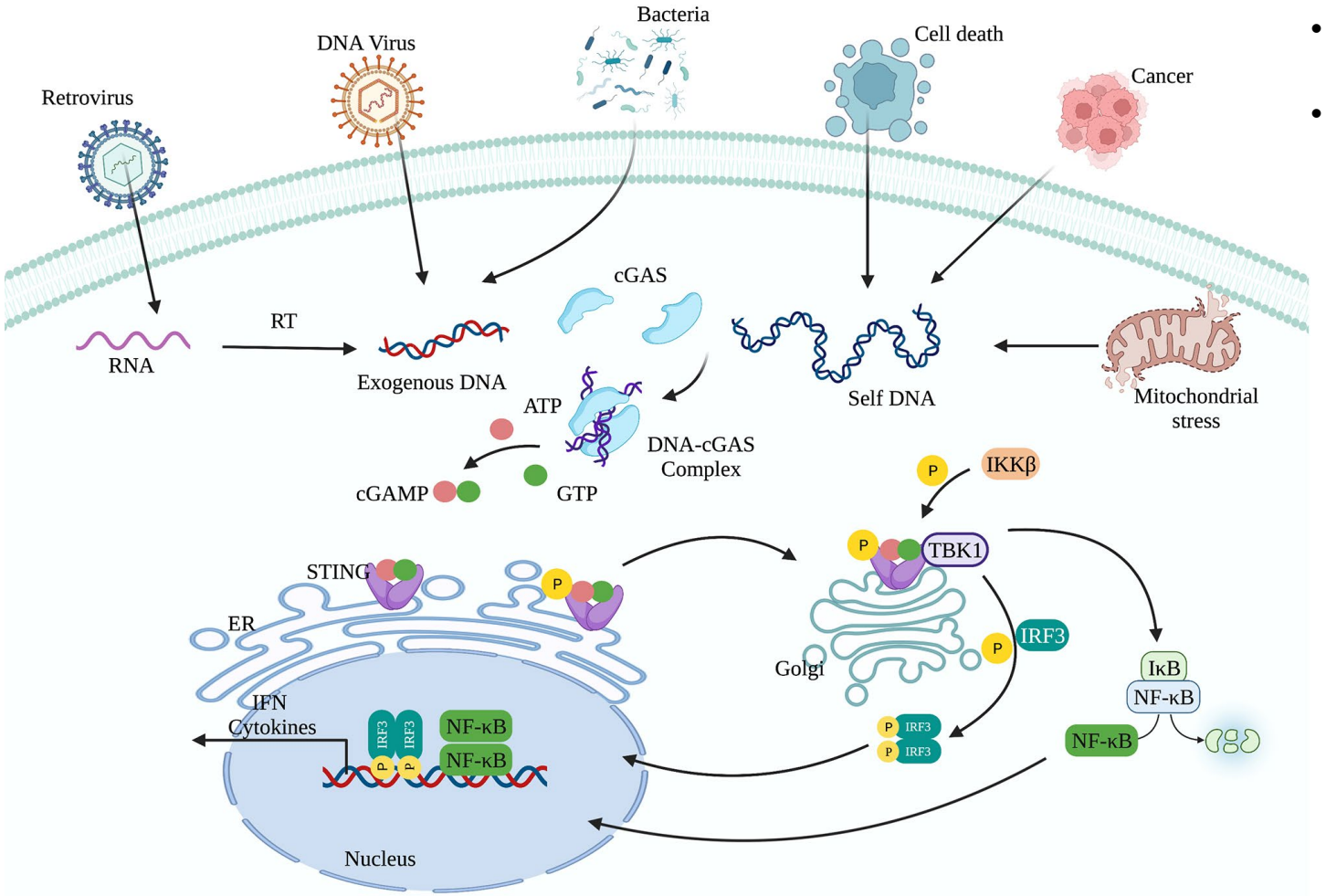
DR PARGi



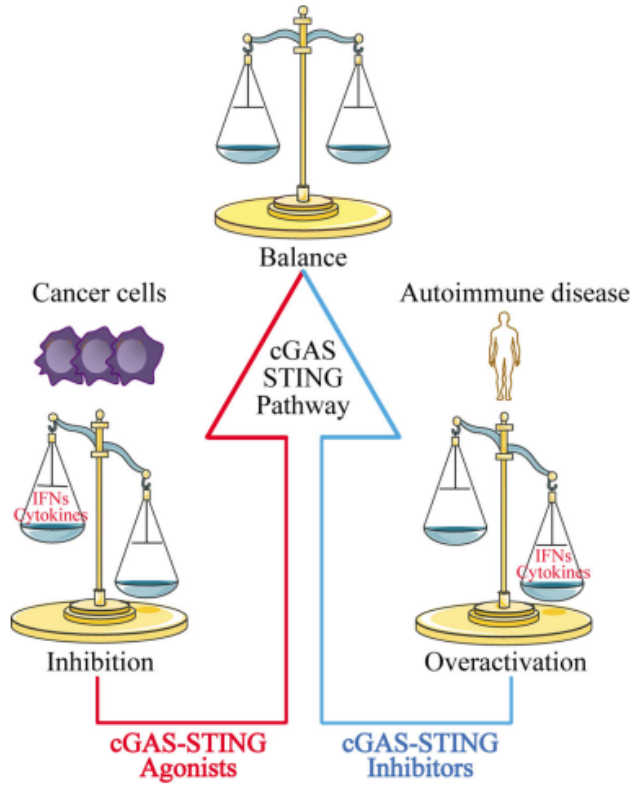
Contents

The cGAS / STING signaling pathway

A balance for oncology and auto-immunity



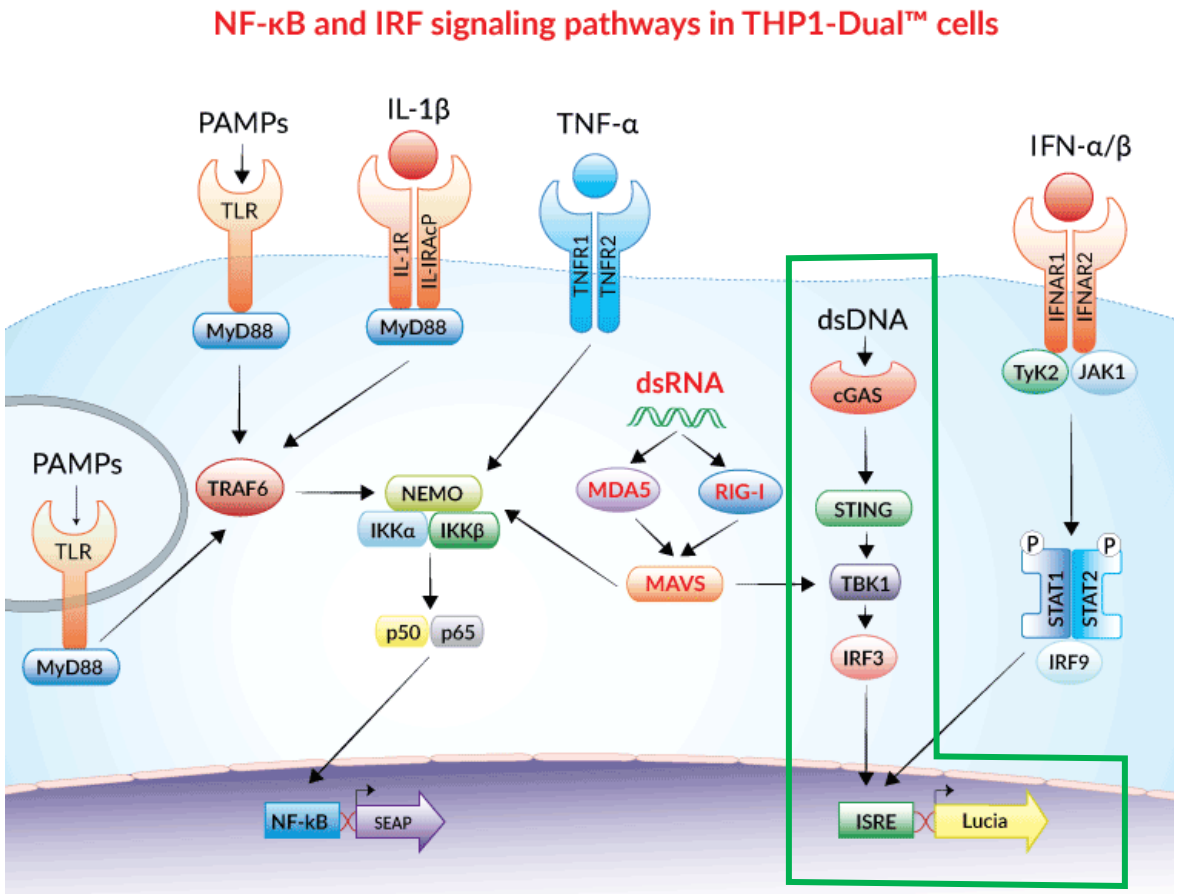
- **Activation of cGAS/STING pathway by DNA initiates the expression of immune regulatory factors.**
- **Targeting the cGAS-STING signaling pathway : a strategy for disease treatment**



Wang et al., Frontiers in Immunology, 2022

Assays developped with THP1 dual™ cells (from Invivogen)

NF-κB-SEAP and IRF-Lucia luciferase Reporter Monocyte



Cells :

THP1-Dual™ cells : Lucia luciferase reporter gene under the control of a interferon-stimulated gene minimal promoter

THP1-Dual™ cells KO for different molecules of the cGAS/STING pathway available: i.e cGAS KO, TREX1 KO, STING KO, IRF3 KO, TBK1 KO..

2 assays developed at Evotec:

- Stimulation of the IFN pathway by transfection using VACV70, a viral oligonucleotide (48h)
- Stimulation of the IFN pathway by cGAMP, a STING agonist, in presence of lyovec (24h)

Read out :

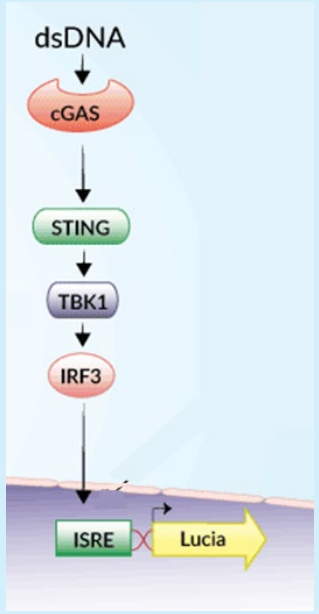
- Luminescence detection with QuantiLUC reagent (Luciferase substrate) - Pherastar
- CellTiterFluor or for viability assay

Activation of the STING pathway by dsDNA transfection

THP1 assay with VACV70 transfection

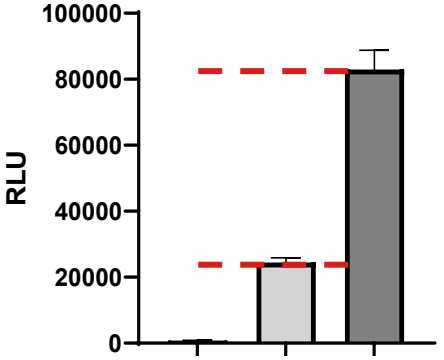
- **Background:** Stimulation of the IFN pathway by transfection using VACV70, a viral oligonucleotide, at a sub-optimal dose to evaluate compounds able to increase this response

- **Experimental conditions:**
  - 384 well plate format
  - 15 000 cells / well
  - Compound pretreatment (0.5% DMSO)
  - 0.5 ug/ml VACV70 (in Lyovec)
  - Incubation 48 hours luminescence
  - detection of the luciferase in THP1 supernatants



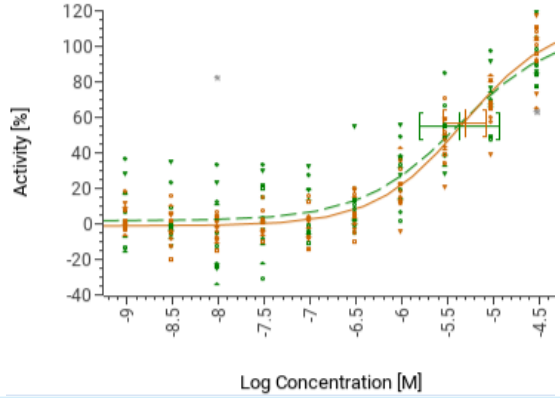
- **Outcome:**
  - dose-dependent activation of the IFN pathway by drugs
  - Normalization on cells transfected with VACV70 in absence of compounds (0% compound activity) and with the referent compound at the top concentration (100% activity)

Window to measure the cpds activity



THP1 cells	+	+	+
VACV70 transfection	-	+	+
Ref Cpd top concentration	-	-	+

Compound dose response



Assessment of STING pathway inhibitors in a primary cellular assay

A luciferase reporter-based cellular assay

Aim

Profile STING inhibitors in a luciferase reporter-based cellular assay

Experimental setup

- THP-1-Dual cell line
- Stimulation with cGAMP in the presence of LyoVec
- Measuring Luciferase activity

Outcome

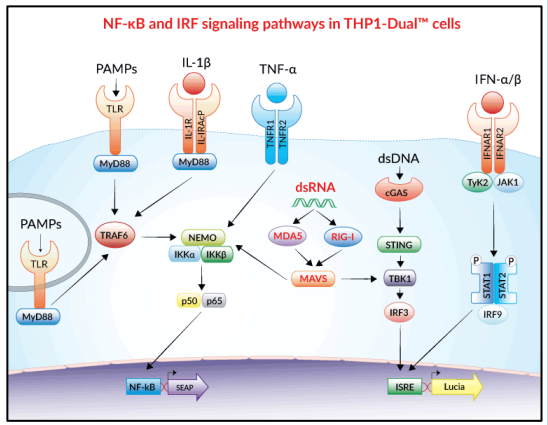
- **Assay performance**
  - Very good assay window. Highest assay window when the LyoVec transfecting reagent is added to cGAMP (S/B=71)
  - Z'>0.6
- **Compound profiling**
  - Suitable cell line model for profiling compounds such as STING inhibitors

WORKFLOW

THP-1 Dual cell pre-treatment with compounds for 2h → Stimulation of THP-1 Dual cells with cGAMP/LyoVec complex for 24h → Luciferase activity measurement

THP-1-Dual cells

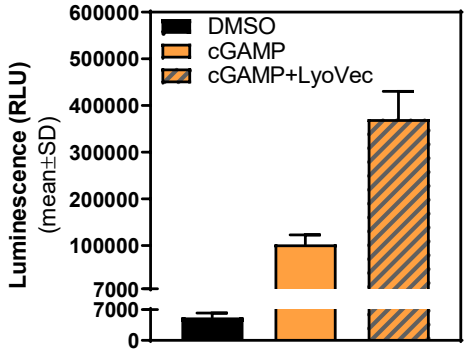
THP-1-Dual cell line (InvivoGen)



https://www.invivogen.com/

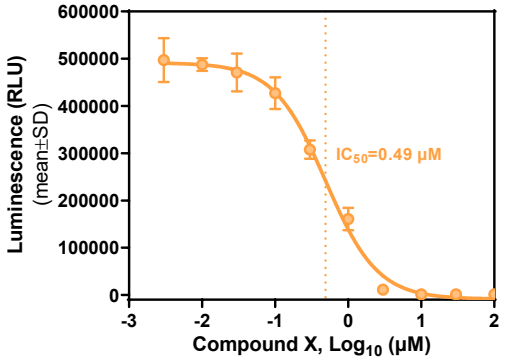
RESULTS

Assay window



	S/B
cGAMP	20
cGAMP+LyoVec	71

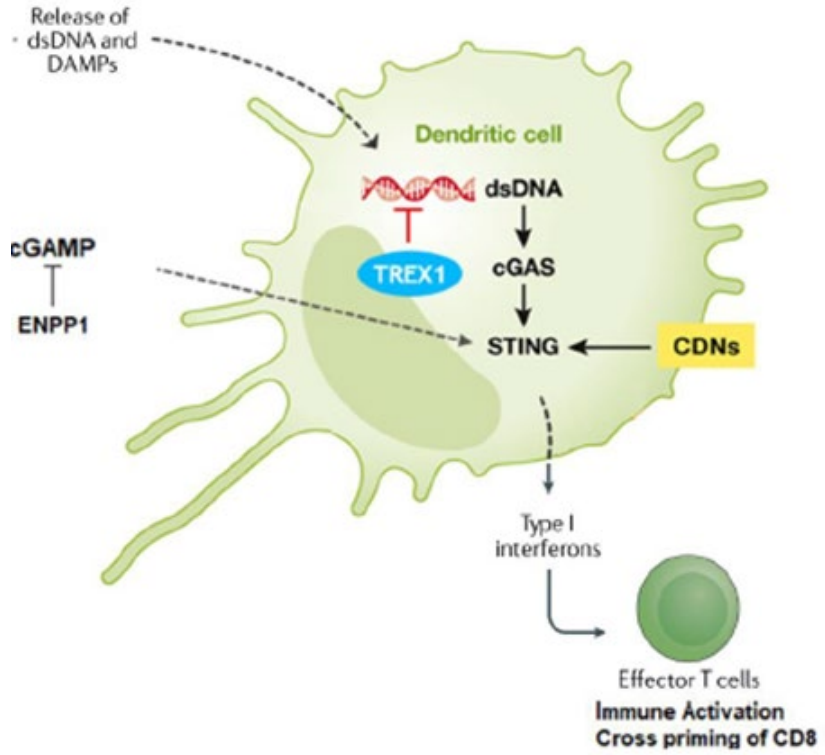
Profiling STING inhibitor



cGAS / STING pathway activity

Assay on primary cells under development

- **Background:** Stimulation of the the cGAS/STING IFN pathway by transfection using dsDNA, at a sub-optimal dose to evaluate compounds able to increase this response
- **Experimental settings:**
  - Primary cells : PBMC, macrophages or monocytes
  - Identify drug efficacy to activate immune myeloid cells
- **Outcome:** dose-dependent IFN response of primary immune cells
- **Read out under evaluation**:
  - Cytokines production : IFN-β, CXCL10, IFN-α, IL12, TNF-α, IL6, IL1β, IL18, INFα, IFNγ
  - mRNA analysis: ISG genes
  - Protein phosphorylation
    - cGAS / STING pathway: pSTING, pIRF3
    - IFN signaling : pSTAT1

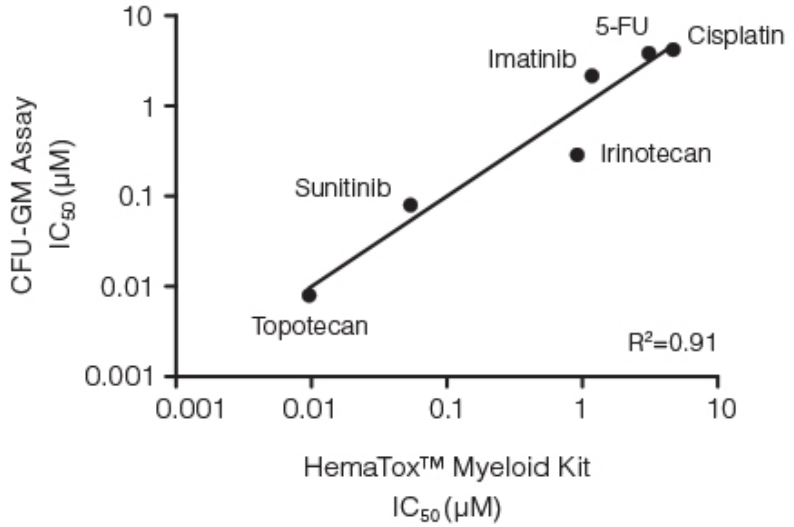


Contents

HemaTox Assay

HemaTox™ Kits for Lineage-Specific Toxicity Testing

- **HemaTox™ kits** (Stemcell Technologies) have been developed for testing the effects of drugs on the growth and lineage-specific differentiation of human hematopoietic stem and progenitor cells (HSPCs)
  - Each kit tests for the effect of drugs on the outgrowth of specific progenitor cell lineages, from CD34+ cells isolated from cord blood (CB) or bone marrow (BM)
  - The HemaTox™ Erythroid Kit , HemaTox™ Myeloid Kit and HemaTox™ Megakaryocyte Kit are designed to test erythroid, myeloid and megakaryocyte-specific toxicity, respectively
- https://www.stemcell.com/products/brands/hematox-in-vitro-hematotoxicity-testing.html
- HemTox is implemented at Evotec in a 384-Well plate format



Correlation Between IC50 Values for Six Drugs Measured Using the CFU-GM Assay and the 96-Well Plate Liquid Culture-Based HemaTox™ Myeloid Kit (Stemcell Thechnologies)

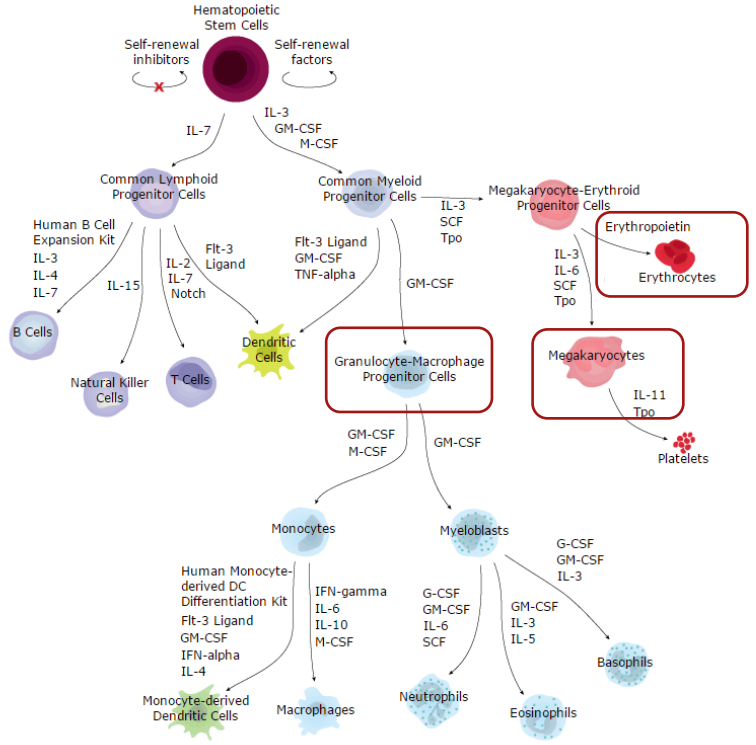
HemaTox Assay

Description

- Differentiation of different HSC progenitors
  - Erythroid kit (7 days);
    - CD71
    - GlyA
  - Megakaryocyte kit (10 days);
    - CD41
    - CD45
  - Myeloid kit (7 days);
    - CD15
    - CD13

- Quantify the proliferation and differentiation of human hematopoietic stem cells;
- Quantification of absolute cell numbers by flow cytometry;
- High throughput capacity (384-well format);
- Including the senescence marker p21;

Hematopoietic Stem Cell Differentiation Pathways & Lineage-specific Markers



Contents

Subcutaneous BRCA2 deficient or proficient DLD1 xenograft model

PK/PD studies of inhibitor as single agent

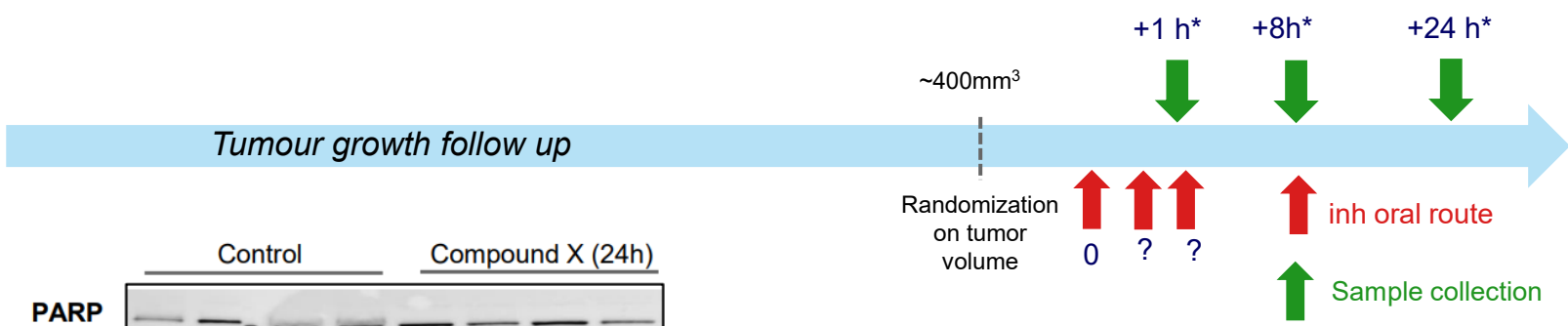
Objective:

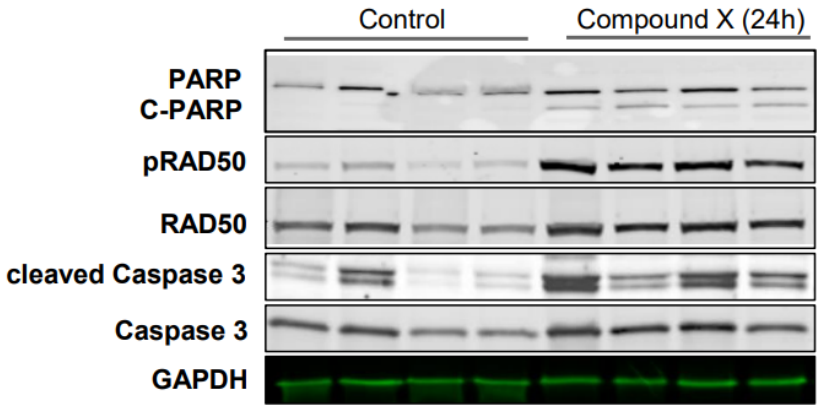
- Assess the concentration of inhibitor after a single oral administration in blood, tumour and a surrogate tissue (eg Spleen)
- Assess PD markers modulation in tumors

Study design :

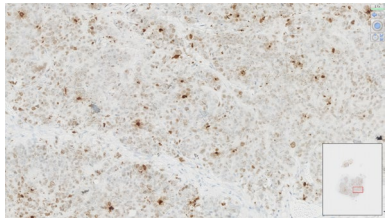
Female Nude mice s.c. injected with DLD1 BRCA2 deficient or proficient cells

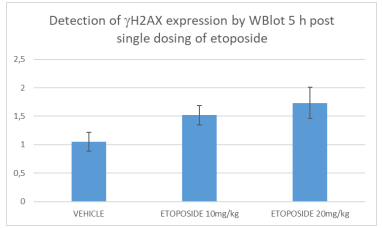
s.c. injection of DLD1-BRCA2$^{-/-}$ $^{or+/+}$ cells





γH2AX foci in DLD1 BRCA2$^{-/-}$ tumor





End point:

Blood collection

- Compound concentration (Bioanalysis)

Tumour sampling

- Compound concentration (Bioanalysis)

Spleen

- Compound concentration (Bioanalysis)

PD markers:

- γH2AX expression by ELISA or WBlot and IHC
- C-PARP by WBlot
- C-Casp3 by Wblot
- Potential for pRAD50, pKAP1, pRPA

* Sampling times will be confirmed after PK study in mice

In vivo evaluation for DDR drug discovery

Mechanism of action,, PK/PD relationship, tolerability and efficacy in tumour models

Compounds with **suitable in vivo PK properties** and bespoke formulation dosed (oral gavage/I.V)

- PK/PD assessment in e.g. BRCA2 -/- or +/+ DLDL1 xenograft model
- DDR agent administered as single agent

- Evaluate **tolerability** after repeated dosing in non-tumour bearing mice to select tolerated dose and schedules
  - Sample organs and blood

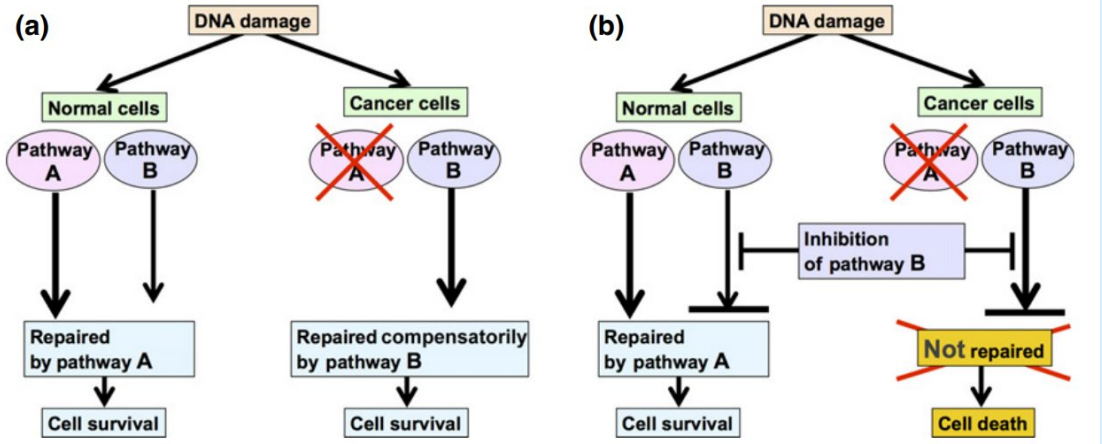
- Assess **antitumor efficacy and therapeutic window of selected cpds** in tumor-bearing mice
  - Sample tumors and blood at end of study to measure PD markers, blood cell count and compound concentration
    - mouse models :
      - Xenograft DLD1
      - Syngeneic B16 (melanoma) and MCA205(fibrosarcoma)
  - Inhibitors as singe agent in DLD1 model and in combination with ICT in B16 model

Contents

Synthetic lethality phenotype analysis

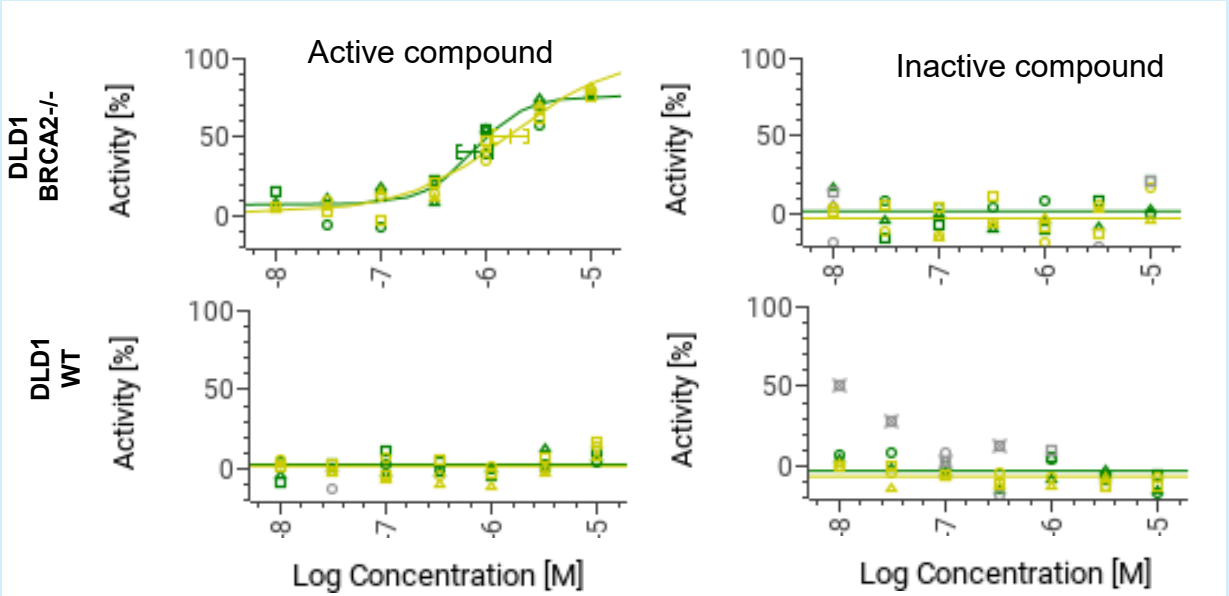
Targeting DNA repair pathway in DNA repair deficient background

- **Rationale:** DNA damage are detected and repaired by different specific and redundant pathways. (a)Inactivation of one pathway (pathway A) will be compensated by a second pathway B. (b) Targeting DNA repair in cancer cells with mutation in DNA repair pathway to induce synthetic lethality



- **Assay principle:** Quantify of cell survival in different cellular background (ex. homologous recombination inactive (Brca2-/-), NHEJ inactive (XRCC4-/-)) ;
- **Read out of viability**: Cell titre Glo® or Incucyte®
- **Throughput:**
  - 27 compounds in dose response
  - Suitable for Tier1 assay

Case study: Monitoring synthetic lethality of HR deficient cell line (pathway A) with compound targeting pathway B.
Measurement of cell viability by CTG after 6-days of exposure to compound, % of activity normalized to staurosporine induced cell death.



Active compound induces cell death in a dose response manner in HR deficient cell line (DLD1 BRCA2-/-) but not in isogenic HR proficient cell line (DLD1 wt)

Hosoya and Miyagawa Cancer Science 2014

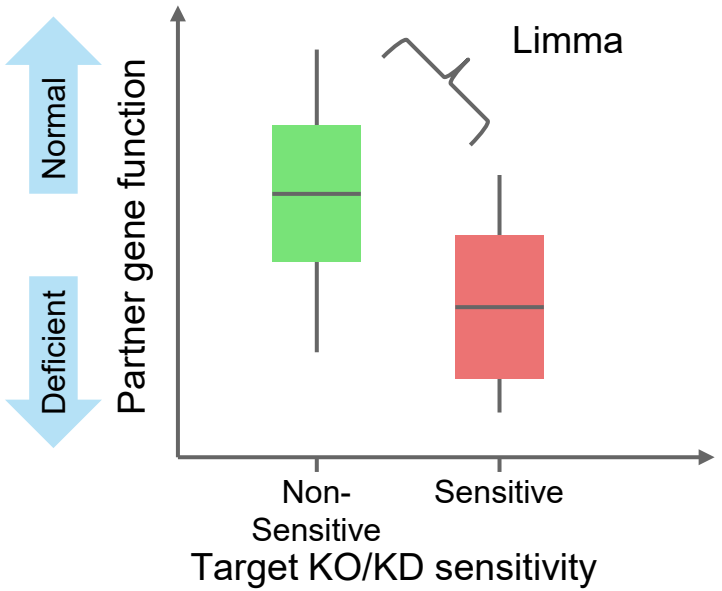
Searching for associations between gene deficiencies and target dependency

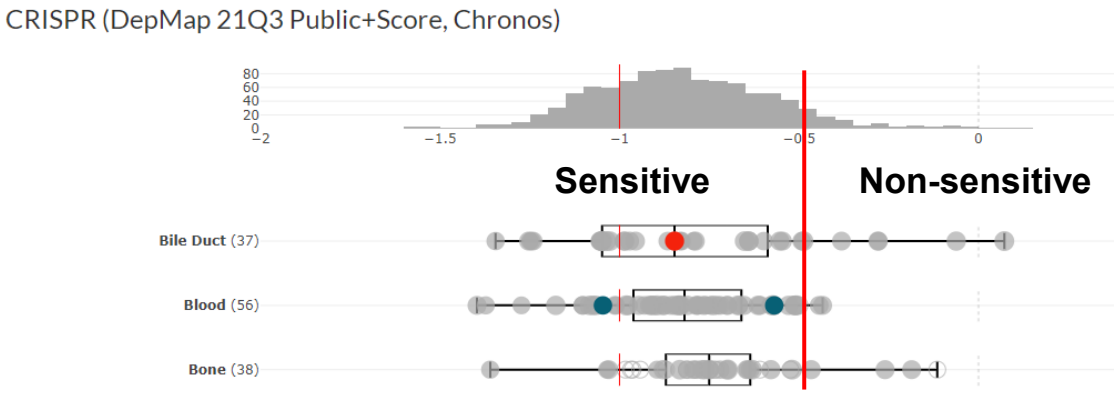
Direct approach

Direct approach:

Comparison between cell lines that are sensitive to the target KO/KD (DepMap CRISPR or RNAi) with non-sensitive cell lines, search for differentially *under*-expressed genes, *over*-deleted genes and enrichment in *over*-mutated genes.

Convenient and fast analysis for gene expression (limma, DeSeq2), not optimal for mutation analysis (requires Chi-square test between categorical mutation status and categorical sensitivity status)





Example from DepMap for a target of interest: split the cell line into two groups (threshold: -0.5), then perform comparisons

Searching for associations between gene deficiencies and target dependency

Reverse approach

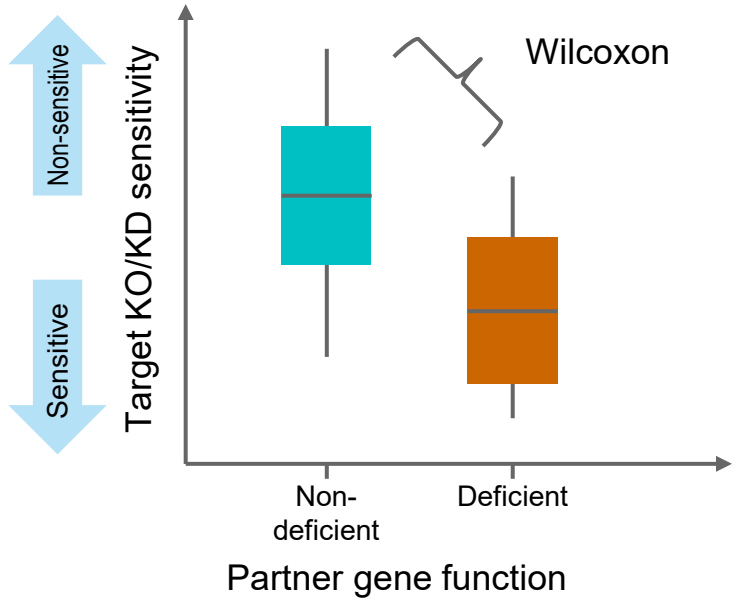
Reverse approach:

Comparison between cell lines that have a deficiency in partner gene (*under*-expressed, *over*-deleted and *over*-mutated) with non-deficient cell lines, and search cases where deficient cell lines are more sensitive to the target KO/KD than the non-deficient cell lines.

Multiple definitions of deficiencies which require definitions of thresholds

- gene expression levels, CNV levels
- mutation types – pathogenicity, Loss of function vs. gain of function, etc.

The approach is more resource consuming but more sensitive for mutation data (preferred Wilcoxon test between categorical mutation status and numeric sensitivity values)



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