

Cecilia Lundin¹, Bastiaan Evers¹, Daniel Ebner², Christina Bauerschmidt³, Timea Palmai-Pallag³, Tomohiro Yoshikawa⁴, David Walker⁴, Alun McCarthy⁴, Thomas Helleday^{1,3}

¹Science for Life Laboratory, Stockholm University, Stockholm, Sweden; ²Target Discovery Institute, Oxford University, Oxford, UK; ³Gray Institute for Radiation Oncology and Biology, Oxford University, Oxford, UK; ⁴PharmacoGenomic Innovative Solutions, Cytopathfinder, UK/Japan

Introduction:

- Homologous recombination is important in resolving replication fork lesions and repair double-strand breaks in the S/G2 phase of the cell cycle
- Oncogene-activation in cells can cause replication stress leading to replication-associated DNA lesions
- Understanding the role of different HR proteins in repair of DSBs and replication lesions may identify potential targets for use in anticancer treatment in combination with existing treatment or as monotherapy utilizing cancer-specific lesions

'Aim of this study is to find novel target genes involved in homologous recombination'

Experimental layout:

- A genome-wide siRNA screen conducted in U2OS cells and looking at RAD51 foci response after damage induced by irradiation (IR) as well as spontaneous RAD51 foci levels
- Experimental plates were prepared by Cytopathfinder where a genome-wide siRNA library (Dharmacon) was pre-mixed with transfection reagent (DharmaFect1) and accelerator on 384-well plates
- In total, the genome-wide library consisted of fifty-eight 384-well plates and the screen was run in duplicate, divided up in separate batch runs
- Treatment with either irradiation (IR) to induce double-strand breaks (DSBs) or leave cells to look at the response to spontaneous lesions
- Cell seeding and antibody staining were performed on a automated liquid-handling robot (Janus, Perkin Elmer) and high-content image analysis using InCell Analyzer 1000 (GE Healthcare)
- Cell viability from siRNA depletion measured on untreated plates using the Resazurin assay

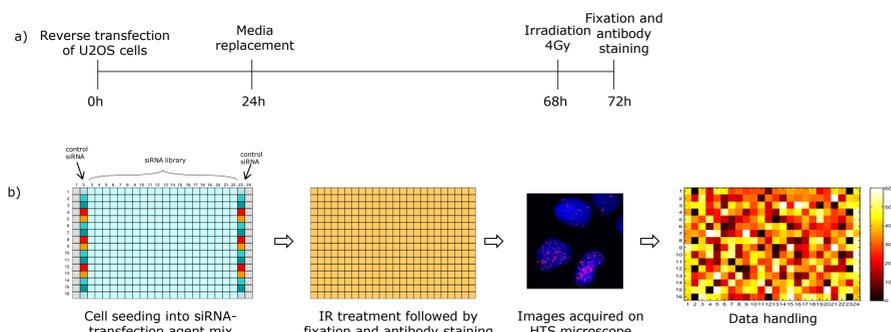


Figure 1 – Experimental screen layout. **a)** Protocol time for genome-wide siRNA screen. **b)** Cells were transfected with siRNA library and then either left untreated (UT) or treated with irradiation (IR) 68h after protein knock-down. At 72h after siRNA transfection, cells are fixed and subsequently stained with antibodies (α -RAD51 and α -rabbit Alexa 488) and nuclear stain (DAPI). Images were acquired on a InCell Analyzer 1000 (GE Healthcare) and analysed using the InCell Investigator software. Final data handling is performed in MatLab.

Results:

- To evaluate quality of screen, for each screen run the Mean foci number of positive and negative control siRNAs were compared (Figure 2a) as well as the correlation between the whole data set for run 1 (GW1) and run 2 (GW2; see Figure 2b)

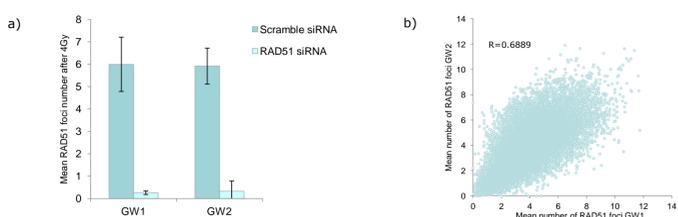


Figure 2 – Evaluation of screen quality. **a)** Mean number of RAD51 foci for control siRNAs from the duplicate runs showing a significant difference in RAD51 foci formation between the positive and negative control siRNAs. The Z-factor for the different plates were in the range of 0.3–0.7. **b)** Correlation of RAD51 foci data from irradiated plates from the duplicate runs. The correlation coefficient $R=0.6889$.

- Screen was divided into two data sets according to treatment (IR- or un-treated set)

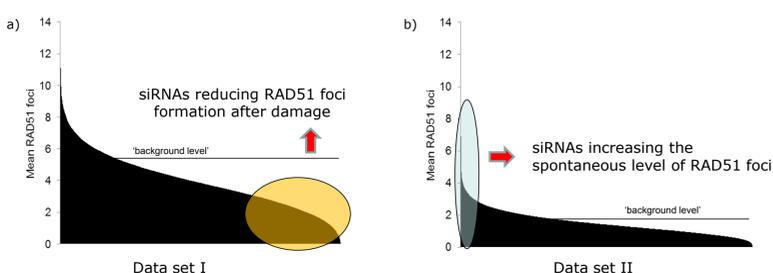


Figure 3 – Data from each screen run was divided into two data sets according to treatment. **a)** Mean number of RAD51 foci in all siRNAs from irradiated plates i.e Data set I. **b)** Spontaneous formation of RAD51 foci in siRNAs from untreated plates i.e Data set II. The different data sets will identify genes that are important either for the RAD51 foci response to IR-induced lesions or genes that might suppress spontaneous induction of homologous recombination.

Results Data set I - genes reducing RAD51 foci formation after IR:

- To aid processing of all data, final data handling was performed using MatLab. This made it possible to compile all data and evaluate any differences in RAD51 foci response that could be due to for example robotic errors

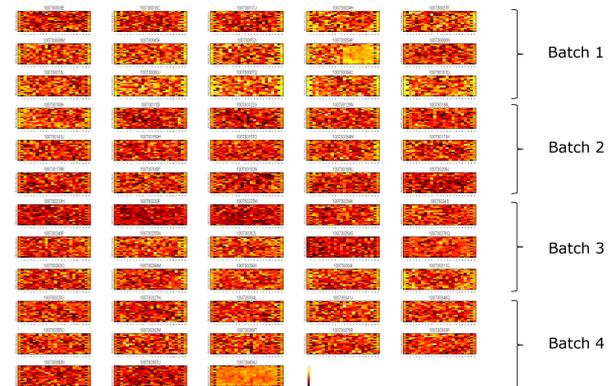


Figure 4 – Heat map of "mean number of RAD51 foci" in irradiated plates. Colour range (dark red to light yellow) goes from 0 to 10 mean number of RAD51 foci. Experiment was run in batches of 15 plates per batch run and overall the variance between plates and batches are low.

- Data was then normalized using a variant of B-score normalization that we have called 3D B-score normalization. Apart from looking at row and column effects within the plates, the 3D B-score also looks at systematic aberrations of whole plates across the dataset, which adds a third dimension to the B-score normalization and will correct for any specific deviations in a specific plate as well.

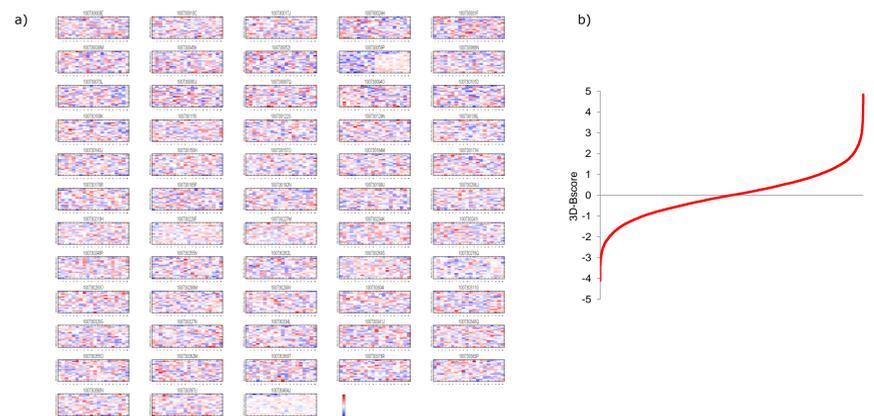


Figure 4 – 3D-Bscore data of irradiated plates. **a)** Heat map of "3D-Bscore" in irradiated plates. Colour range (blue to white to red) goes from -4 to 5. Data is normalized to the mean number of RAD51 foci for whole data set as well as corrected for row, column and plate effects so we can compare results of siRNAs from different plates. **b)** Plot illustrating the span of 3D-Bscores for the whole data set.

- When 3D-Bscores had been assigned to every siRNA, the data was filtered depending on toxicity data, discarding siRNA that are toxic (>70% toxicity in Resazurin assay) and only including siRNAs where at least 100 cells were counted during foci analysis.
- Hit selection was then performed by taking the top 250 hits from the two duplicates and selecting only those that came up in both hit list from the duplicates. Out of the 250 siRNA-list, ~30% was overlapping between the duplicates which gave us a hit list of 67 siRNAs.
- Several known homologous recombination proteins came up as positive hits (e.g. BRCA1, BRCA2 and RPA1) indicating a reliable screen.

Future plans:

- Use gene enrichment analysis to evaluate if hits can be assigned to specific biological characteristics.
- Selected hits will be validated using the same read-out as the primary screen (i.e RAD51 foci response) but using deconvoluted siRNA (4 siRNAs/gene) instead of the pool used in the screen. Genes where >2 of the deconvoluted siRNAs is validated will be regarded as true hits.
- To further evaluate validated hits, different secondary assays will be used, for example studying HR activity using the DR-GFP assay, cell cycle profiles to discard genes that reduce RAD51 foci by arresting cells in G1 as well as confirming protein knock-down with qRT-PCR or Western.
- Processing of Data set II is underway.