University of Southampton | Medicine

Shining a light on the *FCGR* locus; genomic analysis of germline and somatic lesions

Sarah Frampton[1], Jade Forster[2], Marine Armand[1], Helen Parker[1], Khiyam Hussain[3], Chantal Hargreaves[4], Amatta Mirandari[1], Nicolas Carey[1], Daniel Rose[1], Russell Foxall[3], Rosanna Smith[3], Dean Bryant[1], Stephen Beers[3], Mark S Cragg[3], Jane Gibson[1] & Jonathan C Strefford[1]

[1]*Cancer Genomics,* [2] *WISH Laboratory,* [3]*Antibody and Vaccine Group, Cancer Sciences, University of Southampton, UK;*
[4] *NIHR Oxford Biomedical Research Centre, Department of Clinical Immunology, John Radcliffe Hospital, Oxford, UK*

Introduction

- Monoclonal antibody (**mAb**) immunotherapy is becoming standard in the treatment of cancer, particularly for B-cell lymphoma patients, however responses are variable and **relapse** is common. Thus, the molecular mechanisms that underpin resistance needs to be defined.
- mAb efficacy relies on the interaction with the Fc gamma receptors (**FcγRs**), a family of receptors crucial in the immune response (**Fig 1**).
- Therapeutic response is dictated by the activatory to inhibitory (A:I) ratio between the activatory (FcγRIIa, FcγRIIc, FcγRIIIa and FcγRIIIb) and inhibitory (FcγRIIb) low-affinity FcγR receptors.
- The **low-affinity locus** (*FCGR2A*, *FCGR2B*, *FCGR2C*, *FCGR3A* and *FCGR3B)* is highly polymorphic; harbouring copy number variation (**CNV**) and many single nucleotide polymorphisms (**SNPs**) that influence protein function (**Fig 2**).
- Focal somatic amplification of *FCGR2B* have recently been uncovered in lymphoma patients and are associated with inferior outcomes.
- The locus is **extremely challenging** to study due to the **vast sequence homology** and reads from traditional short-read sequencing technologies fail to be adequately aligned or assembled.
- For the first time, an **accurate genomic map** of the *FCGR* locus can be assembled with the use of Oxford Nanopore's long-read technology to elucidate high-resolution sequence, breakpoint, phasing and base modification information.

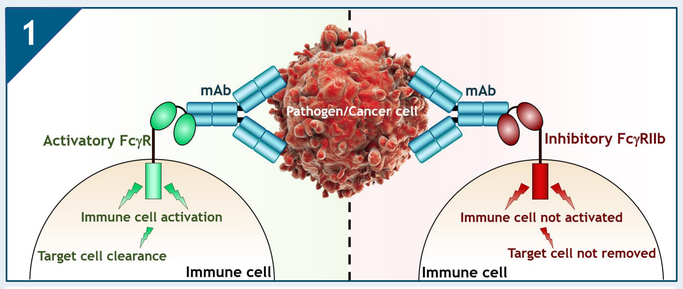

Figure 1 The FcγRs role in the removal of pathogens/malignant cells through the engagement with IgG/mAbs that have opsonised the target. The predominant mechanisms involve antibody-dependent cellular cytotoxicity, antibody-dependent cellular phagocytosis and complement-dependent cytotoxicity. Precise regulation of the activatory FcγRs vs the inhibitory FcγRIIb permits the flexibility and fine-tuning necessary for an appropriate immune response whilst avoiding autoimmunity.

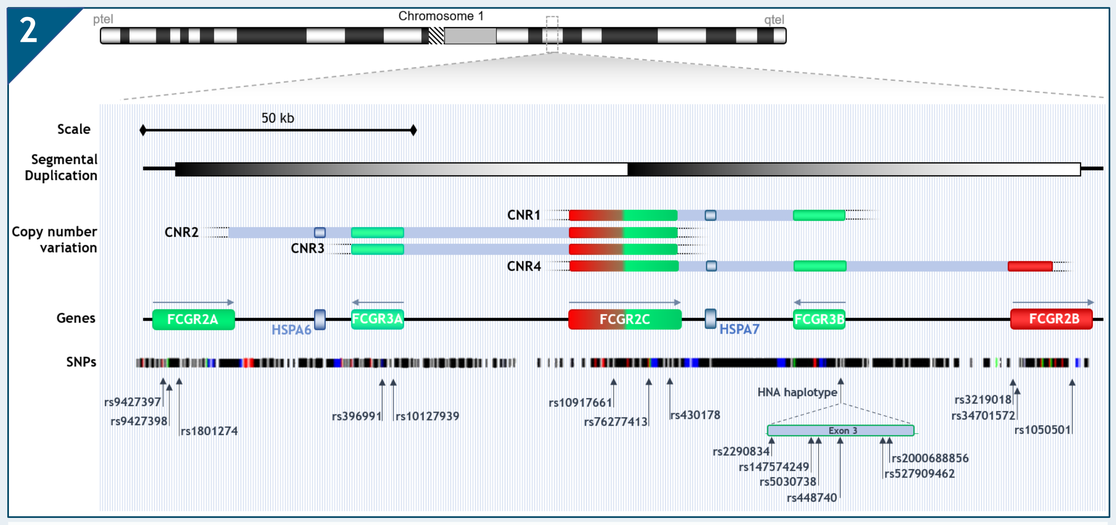

Figure 2 The current understanding of FcγR genomic architecture. The low-affinity FcγR genes are encoded in a 200kb locus on chromosome 1q23.3, which encompasses two tandem 85kb paralogous blocks derived from multiple ancestral recombination and segmental duplication events. *FCGR2C* is a fusion gene that encodes FcγRIIc, a receptor that shares intracellular activatory signalling similar to FcγRIIa but an extracellular domain almost identical to FcγRIIb. The locus is extensively polymorphic and SNPs thought to be relevant to the efficacy of mAb immunotherapy are highlighted. CNV consistently occurs in regions containing a series of genes, termed CNV regions (**CNRs**), but their precise boundaries are unknown.

Aims

1. Generate **highly-detailed accurate maps** of the low-affinity *FCGR* locus under different CNV states
2. Improve genomic/epigenetic understanding of *FCGR* region and it's **regulation**
3. Provide reference maps for **further omics** studies into **normal and malignant** FcγR **regulation**

Preliminary Results

Nanopore WGS

WGS optimisation show the **ligation library prep** and **flowcell flush** steps are substantially beneficial to both read length and throughput (**Fig 4a-c**). Nanopore long-reads have the capacity to **uniquely map** to the *FCGR* locus in the current human reference where short-reads have previously failed (**Fig 4d**).

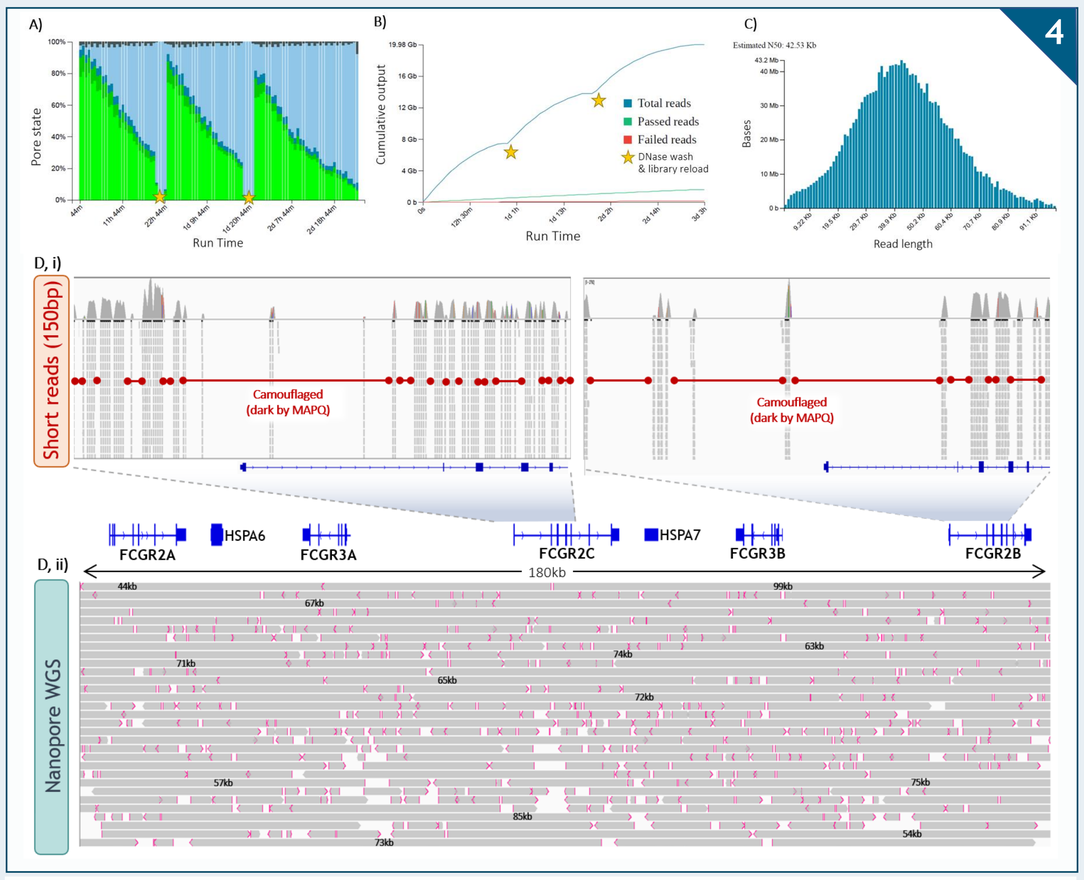

Figure 4 Key results from the WGS optimization performed to help guide the development of the enrichment approaches. **A-B)** Real-time analysis plots produced by MinKNOW, demonstrates how flushing the flowcell with DNaseI and reloading library can further increase the throughput from one flowcell as it 're-activates' more pores for further sequencing (light green). **C)** High read length N50 achieved with the ligation prep **D)** IGV visualization of the difference in mapping quality to the hg38 reference genome between short-reads **(i)** and nanopore long-reads **(ii)**. Dark grey bars indicate sequencing reads with a high mapping quality (MAPQ). Reads with a low MAPQ have been removed, leaving a significant proportion of the *FCGR* locus to not be covered (camouflaged) by the short-reads. Read lengths have been highlighted on some of the nanopore reads to give an indication of the range.

Enrichment

FCGR enrichment has been performed on 19 samples with **cas9-targeting** and 2 samples with **adaptive sampling** (**Table 1**). Initial analysis has showed **significant enrichment** of the target region resulting in **500-fold** and **6.5-fold** increase in on-target reads respectively.

Table 1 Comparison of input, output and *FCGR* coverage between whole genome sequencing (WGS), WGS with adaptive sampling (AS) and cas9-targeting

Library prep	Sequencing runs	Samples sequenced	Total yield (Gb)	Read N50 (kb)	Mean read quality	Average *FCGR* coverage
WGS	34	3	234.5	26.0	15.4	9.5x
WGS + AS	7	2	21.6	28.7	14.4	46.2x
Cas9	38	19	61.7	27.3	14.9	201.8x

Preliminary analysis

Initial analysis of the nanopore data has revealed it is possible to successfully phase across the entirety of the *FCGR* locus (**Fig 5a**), confidently detect single nucleotide variants (**Fig 5b**) and identify methylation patterns (**Fig 5c**); thus facilitating the exciting opportunity to **overcome the inherent difficulties** of sequence homology.

CANCER RESEARCH UK

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Contact
Sarah Frampton | PhD candidate
Email: smf1g18@soton.ac.uk
: @smframptonsoto1

Methods

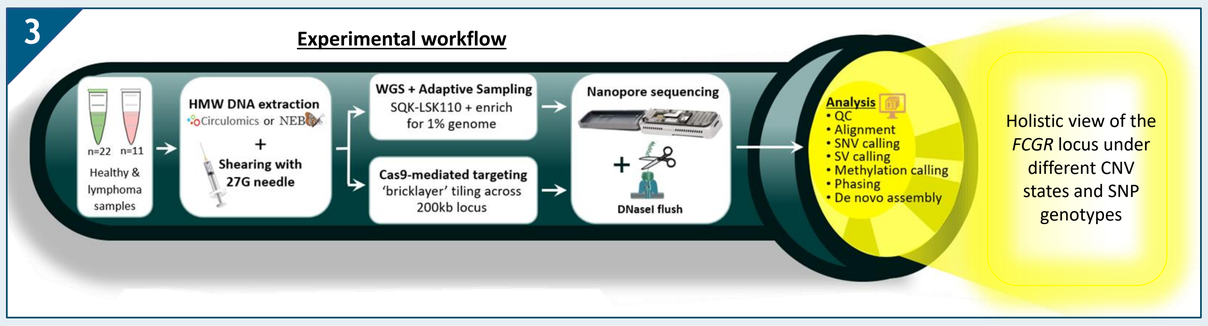

Figure 3 Experimental and analysis workflow. A cohort (n=271) of healthy donor peripheral blood mononuclear cell (**PBMC**) samples have all undergone **extensive molecular characterisation** with MLPA, Taqman genotyping and Haloplex^{HS} to best define *FCGR* CNV and SNP structures. **22** have been selected for this project that display a range of genomic complexity and different *FCGR* CNV states. Additionally, both germline and **malignant** material is available from a fresh-frozen lymphoma cohort (**n=11**) to investigate somatic changes. So far, 5 samples have been processed with a WGS approach and 19 with the cas9-targeting on **R9.4.1 flowcells**. As sequencing is only performed on **native DNA**, additional insight into DNA modifications also exists ready to analyse. Data will be analysed using a bioinformatics pipeline to examine sequence polymorphisms, structural variation, breakpoint, phasing definition and the epigenetics of the *FCGR* locus.

Preliminary Results and Future Work

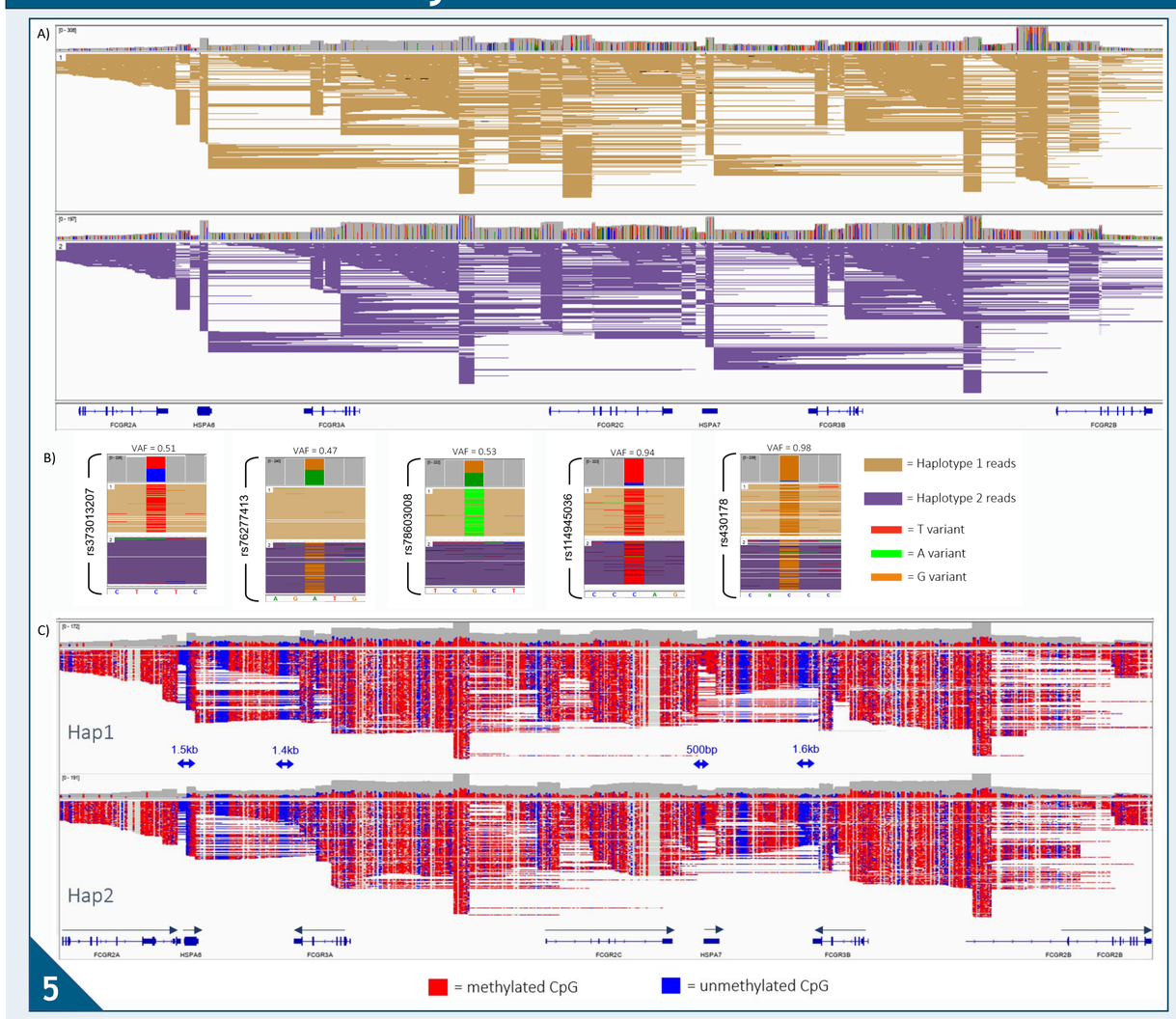

Figure 5 IGV visualisations of long-read preliminary analysis from combined WGS and cas9 data from one sample **A)** Phased coverage across the entire *FCGR* locus in 1 haploblock, reads from haplotype 1 and haplotype 2 are coloured in gold and purple respectively. **B)** Haplotype-resolved view of SNPs. Variant bases coloured differently from the gold/purple of the read that is aligned to the reference along the bottom. These 5 SNPs are of interest as have been reported to be involved in *FCGR2C* alternative splicing. Variant allele frequency (VAF) calculated using NanoPolish's signal-level probabilistic model. **C)** Haplotype-resolved 5-methylcytosine patterning across whole locus. Arrows near reference genes represent which direction genes are read in. Blue arrows with labels highlight size of some unmethylated regions.

- One expected outcome from this work is the generation of high-resolution reference maps of the low-affinity *FCGR* locus under different SNP genotype and CNV states in normal cells.
- Using the same approaches to **characterise primary lymphoma samples** we aim to generate a detailed understanding of the somatic lesions in the *FCGR* locus and their **functional impact**.

Discussion

Nanopore sequencing presents, for the first time, the possibility to overcome the inherent challenges of **the highly homologous** nature of the *FCGR* locus, facilitating a complete view of the region and it's regulatory control. This in turn will enhance further **multi-omics** work on the FcγRs by the wider scientific community, providing them with a unique resource that enables the interrogation of **gene expression** and **epigenetic modifications** that control the system. In broader terms, this work will catalyse a detailed understanding of FcγR **regulation** and ultimately improve **risk-adapted stratification** of cancer patients. Detailed knowledge of the molecular mechanisms that reinforce mAb resistance will promote the development of novel therapeutics to augment FcγR function and **improve response** to **mAb immunotherapy**.