Capture Hi-C reveals novel candidate genes and complex long-range interactions with related autoimmune risk loci

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Genome-wide association studies have been tremendously successful in identifying genetic variants associated with complex diseases. The majority of association signals are intergenic and evidence is accumulating that a high proportion of signals lie in enhancer regions. We use Capture Hi-C to investigate, for the first time, the interactions between associated variants for four autoimmune diseases and their functional targets in B- and T-cell lines. Here we report numerous looping interactions and provide evidence that only a minority of interactions are common to both B- and T-cell lines, suggesting interactions may be highly cell-type specific; some disease-associated SNPs do not interact with the nearest gene but with more compelling candidate genes (for example, \textit{FOXO1}, \textit{AZI2}) often situated several megabases away; and finally, regions associated with different autoimmune diseases interact with each other and the same promoter suggesting common autoimmune gene targets (for example, \textit{PTPRC}, \textit{DEXI} and \textit{ZFP36L1}).

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The identification of the precise gene targets of variants associated with complex traits detected through genome-wide association studies (GWAS) has proved challenging but is essential if the full potential of genetic studies is to be realised. Accumulating evidence suggests the majority of these variants lie outside traditional protein-coding genes and are enriched in enhancer regions, which are both cell-type and stimulus specific. The task now is to identify which genes are implicated and understand which cell types are involved, to ascertain the biological pathways that are perturbed in individuals who are genetically susceptible to disease. It is well-established that enhancers regulate gene transcription by physical interactions. These can operate over large genetic distances, so the tradition of annotating GWAS hits with the closest, or most biologically plausible gene candidate, may prove misleading and result in expensive, time consuming efforts to define the function of non-causal genes.

The utility of chromosome conformation capture technology (Capture Hi-C) to detect the patterns of interactions between chromosomal regions has been demonstrated. Here, for the first time, we used this approach to characterize the interactions of confirmed susceptibility loci for four autoimmune diseases: rheumatoid arthritis (RA), type 1 diabetes (T1D), psoriatic arthritis (PsA) and juvenile idiopathic arthritis (JIA) with the aim of linking disease-associated SNPs with disease-causing genes. Uniquely, we have tested the interactions in two complementary experiments: first, Region Capture targets regions associated with disease; second, Promoter Capture provides independent validation through capturing all known promoters within 500 kb of lead disease-associated single nucleotide polymorphisms (SNPs). Our study expands on previous applications of the Capture Hi-C method firstly, Region Capture targets chromosomal regions has been demonstrated; second, Promoter Capture determines a complex array of interactions between disease-associated regions and promoters (Fig. 1). After quality control, in the Region Capture experiment, 60.9 million and 54.9 million unique di-tags (comprising one restriction fragment from a capture target region and its ligated interacting partner) were on-target for GM12878 and Jurkat cell lines, respectively (average 21,170 reads per HindIII restriction fragment; 62% capture efficiency). Similarly, in the Promoter Capture experiment, 121.1 million (GM12878) and 115 million (Jurkat) unique di-tags were on-target (average 21,448 reads per HindIII restriction fragment; 70% capture efficiency) (Fig. 2).

At any given false discovery rate (FDR) threshold, interactions are called with an unknown rate of false negatives. With the assumption that interactions called in both the Region and Promoter Capture experiments are more likely to be true positives compared with those only seen in one experiment, we evaluated several potential FDR thresholds (Fig. 3). We saw a consistent

**Results**

**Summary of identified interactions.** Our unique study design determined a complex array of interactions between disease-associated regions and promoters (Fig. 1). After quality control, in the Region Capture experiment, 60.9 million and 54.9 million unique di-tags (comprising one restriction fragment from a capture target region and its ligated interacting partner) were on-target for GM12878 and Jurkat cell lines, respectively (average 21,170 reads per HindIII restriction fragment; 62% capture efficiency). Similarly, in the Promoter Capture experiment, 121.1 million (GM12878) and 115 million (Jurkat) unique di-tags were on-target (average 21,448 reads per HindIII restriction fragment; 70% capture efficiency) (Fig. 2).

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enrichment in interactions called in both experiments at decreasing Promoter Capture experiment FDR thresholds, providing confidence that they represent true interactions. At 5% FDR, we called 8,594 interactions in the Region Capture experiment (Supplementary Fig. 1a) and interactions in the 5C ENCODE (https://www.encodeproject.org/)17 experiments at two regions: IFNAR1 and ILS5 (Supplementary Fig. 1b,c).

Interactions with novel candidate genes. Confirmed interactions provided examples of disease-associated SNPs that do not interact with the nearest gene, but rather with promoters some distance away, implicating entirely different target genes. For example, strong evidence was found to suggest that regions with SNPs associated with RA, situated proximal to the EOMES gene, make strong physical contact with the promoter of AZI2, a gene involved in NFκB activation, some 640 kb away (Fig. 4a) in both GM12878 and Jurkat cell lines. In addition, variants associated with RA and JIA in the 3′ intronic region of COG6, a gene encoding a component of Golgi apparatus, show interactions with the promoter of the FOXO1 gene, mapping over 1 Mb away, in both cell types (Fig. 4b). Recent findings suggest that the FOXO1 gene is important in the survival of fibroblast-like synoviocytes (FLS) in RA18 and is hypermethylated in RA FLS compared with osteoarthritis FLS19, providing strong supporting functional evidence as to gene candidature.

Common interaction targets mediated by multiple genetic loci. Perhaps the most striking finding comes from genetic regions that harbour susceptibility loci for different autoimmune diseases, where the lead disease-associated SNP for one disease maps some distance from the lead disease-associated SNP for other autoimmune diseases; previously, using the ‘nearest candidate gene’ annotation method, different genes would have been assigned to the diseases but our work shows that they may all act on the same gene promoter. We provide three examples below to illustrate the findings. First, the 16p13 region contains SNPs on the same gene promoter. We provide three examples below to illustrate the findings. First, the 16p13 region contains SNPs assigned to the diseases but our work shows that they may all act on the same gene promoter. We provide three examples below to illustrate the findings. First, the 16p13 region contains SNPs

Figure 2 | Flowchart summarizing capture Hi-C experiments by cell line. The ‘Region Capture’ experiment is shown on the left and the ‘Promoter Capture’ experiment on the right. Flowchart sections are coloured by cell type: light blue—GM12878 cells; light grey—Jurkat cells and grey—both cell types. Each section label is shown in dark blue.

Figure 3 | Fold enrichment. Fold enrichment of retained interactions called in the promoter capture experiments with decreasing FDR thresholds, given they had been called in the region capture experiments at the FDR threshold shown. ‘—’ shows the enrichment found by focusing only on interactions called in the region capture experiments for which the other end lay in a HindIII restriction fragment targeted in the promoter capture design.

the HBA locus16 (Supplementary Fig. 1a) and interactions in the 5C ENCODE (https://www.encodeproject.org/)17 experiments at two regions: IFNAR1 and ILS5 (Supplementary Fig. 1b,c).
promoter (Fig. 5a). Furthermore, a region proximal to the \( ZC3H7A \) gene, associated with RA susceptibility, some 1.2 Mb from \( DEXI \), interacts with both the TID/JIA-associated region and the \( DEXI \) promoter.

The second example is provided by RA-associated variants mapping within a strong enhancer region intrinsic to \( RAD51B \), where a significant interaction is observed with the promoter of the \( ZFPM3L1 \) gene. SNPs in the promoter region of \( ZFPM3L1 \) are independently associated with JIA but not RA; however, the interaction of the \( ZFPM3L1 \) promoter with the RA-associated SNPs suggests that the causal gene in both diseases may be \( ZFPM3L1 \) rather than \( RAD51B \). \( ZFPM3L1 \) is a zinc finger transcription factor involved in the transition of B cells to plasma cells and it is noteworthy that the interaction with the RA-associated region was only seen in the B-cell line (Fig. 5b).

Finally we show evidence that SNPs associated with PsA within the \( DENND1B \) gene make strong contact with a region associated with RA within the \( PTPRC \) gene, which is responsible for T- and B-cell receptor signalling and maps over 1 Mb away (Fig. 5c).

We, like others, have demonstrated a complex relationship between promoters and enhancers, where promoters interact with many enhancers and enhancers interact with many promoters, rarely in a one-to-one relationship (Fig. 1 and Supplementary Table 2). Enhancers containing risk variants for autoimmune diseases can, therefore, ‘meet’ at the same promoters. This challenges the assumption that disease-associated SNPs have to be in close linkage disequilibrium (LD) to have a disease related effect on the same gene. In addition, these findings may well-suggest an evolutionary phylogeny, where polymorphic variants regulating expression of the same gene result in either different autoimmune diseases or different molecular mechanisms resulting in risk of the same disease.

Interactions with previously implicated loci. Among the other 141 confirmed interactions, we observed examples of disease-associated SNPs within the 3’ untranslated region, or within introns of a gene, interacting with the promoter of the same gene (\( STAT4, CDK6 \), Supplementary Fig. 2a,b); disease-associated SNPs within lncRNA interacting with the promoter of genes (\( RBPJ, CDK6 \), Supplementary Fig. 3) and several examples of restriction fragments, proximal to those containing disease-associated SNPs, interacting with promoters some distance away (\( ARID5B, IL2RA, TLE3 \), Supplementary Fig. 4a–c), supporting recent findings that disease-associated SNPs are enriched outside transcription factor-binding sites.

Long-range interactions. Perhaps unexpectedly, ~80% of significant interactions occurred at distances exceeding 500 kb (Supplementary Fig. 5) and interacted with ‘non-promoters’,
reducing the number of interactions available for co-validation in the Promoter and Region Capture experiments (targeted genes in the Promoter Capture not extending that far) and reinforcing the idea that GWAS regions may be involved with complex long-range gene regulation possibly involving multiple enhancer elements. To investigate whether these are likely to be true interactions, we compared results from the largest Hi-C data set on GM12878 cells reported, to date\textsuperscript{21}. Of the 4,607 longer distance interactions (> 500 kb) we called at FDR < 5% in our data, 377 were found at 50 times observed over expected in the independent Hi-C data set (Supplementary Data 1). This provided both strong confirmation of our long-range capture Hi-C results we already co-validated with Promoter and Region Capture (for example, FOXO1, ZFP36L1, Supplementary Fig. 6).
and supports many potentially novel interactions (for example, MMEL1, Supplementary Fig. 7), but detailed examination to confirm these long-range interactions is now required.

**Discussion**

Our targeted Capture Hi-C analyses have identified, for the first time, many long-range interactions between autoimmune risk loci and their putative target genes. Using this methodology we have intriguing data illustrating that regions associated with more than one disease, often some distance apart, interact with the same gene and that associated regions can 'skip' genes to interact with more distant novel candidates. Our results provide new insights into complex disease genetics and changes the way we view the causal genes in disease, with obvious implications for pathway analysis and identification of therapeutic targets. Since we uncovered evidence of cell-specific interactions, the current study is likely to be only the beginning of similar explorations. Further work to characterize functionally the observed interactions, including eQTL studies using a range of cell types and stimulatory conditions, are required to determine how disease-associated SNPs influence the risk of disease, with the aim of better understanding disease aetiology.

**Methods**

**SNP and region associations.** All independent lead disease-associated SNPs for RA were selected from both the fine-mapped ImmunoChip study33 and a trans-ethnic GWAS meta-analysis31. Lead disease-associated SNPs were also added from the ImmunoChip fine mapping studies for JIA13 and Psa12. This resulted in a total of 242 distinct variants associated with one or more of the three diseases after exclusion of HLA-associated SNPs. Associated regions were defined by selecting all SNPs in LD with the lead disease-associated SNP (r² > 0.8; 1000 Genomes phase 1 EUR samples; May 2011). In addition to the SNP associations, credible SNP set regions were defined for both T1D- and RA-associated loci discovered by the ImmunoChip array at a 99% confidence level34. RA loci, as defined from the ImmunoChip analysis, were extended to include the credible SNP region where necessary and overlapping regions were merged using the BEDTools v2.21.0 (ref 22) merge command resulting in 211 associated regions.

**Target enrichment design.** To remain hypothesis free and to validate significant findings, two target enrichments were designed. The first targeted the ‘associated region’ and was called the ‘Region Capture’ set. The second targeted all known gene promoters overlapping the region 500 kb up- and downstream of the lead disease-associated SNP dubbed as the ‘Promoter Capture’ set. Capture oligos (120 bp; 25–65% GC, <3 unknown (N) bases) were designed using a custom Perl script within 400 bp but as close as possible to each end of the targeted HindIII restriction fragments and submitted to the Agilent eArray software (Agilent) for manufacture.

**Region Capture design.** Capture oligonucleotides were designed to all HindIII restriction fragments in each previously defined associated region after excluding those already targeted in the Promoter Capture. Regions were extended by one restriction fragment where there was <500 bp between the restriction site and the region start/end. This resulted in 3,159 restriction fragments in total after merging overlapping regions. Of these, 1,028 failed design, 1,096 had both ends captured and 1,035 had one end captured, producing a target capture of 387.24 kb covering a genomic region of 7.46 Mb (3.5 kb/restriction fragment on average). In addition, a control region, which represents a well-characterized region of long-range interactions, was also included: HBA (174.37 kb genomic; 26 restriction fragments; 6.71 kb restriction/fragment).

**Promoter Capture design.** Promoter Capture target regions were defined as 500 kb up- and downstream of each disease-associated SNP. These regions were further extended to encompass the associated regions where appropriate. HindIII restriction fragments were identified within 500 bp of the transcription start site of all genes mapping to the defined regions (Ensembl release 75: GRCh37) and overlapping regions were merged using the BEDTools27 merge command resulting in 6,296 restriction fragments. Of these, 792 failed design, 2,986 had both ends captured and 2,518 had one end captured, producing a target capture of 1.02 Mb. The 5,504 captured restriction fragments covered a genomic region of 38.76 Mb (7.04 kb/restriction fragment on average) and contained promoters for 3,857 genes. The HBA control region previously mentioned was also included.

**Cell culture and crosslinking.** The GM12878 B-lymphoblastoid cell line, produced from the blood of a female donor with northern and western European ancestry by EBV transformation, was obtained from Coriell Institute for Medical Research. Lymphoblastoid cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 per 20 mM L-glutamine supplemented with 15% foetal bovine serum (FBS) in 25-cm² vented culture flasks at 37 °C per 5% CO2. The B-lymphoblastoid Jurkat E6.1 cell line, originating from the peripheral blood of a 14-year-old boy in the study by Schneider et al.73, was obtained from LGC Standards and cultured in RPMI 1640 per 20 mM L-glutamine supplemented with 25-cm² vented culture flasks at 37 °C/5% CO2. Two libraries were generated for each cell line, the former being diluted to 50% and the latter to 100% to ensure accurate sequencing. Crosslinking reactions were carried out as described in the study by Belton et al.74. Cells were washed in Dulbecco’s Modified Eagle’s medium (DMEM) and then crosslinked with 2% formaldehyde for 10 min at room temperature. The crosslinking reaction was quenched by adding cold 1 M glycine to a final concentration of 0.125 M for 5 min at room temperature, followed by 15 min on ice. Crosslinked cells were washed in ice-cold PBS, the supernatant discarded and the pellets flash-frozen in liquid nitrogen and stored at –80 °C.

**Hi-C library generation.** Cells were thawed on ice and re-suspended in 50 ml freshly prepared ice-cold lysis buffer (10 mM Tris-HCl pH 8, 10 mM NaCl, 0.2% (v/v) Tween 20, 500 mM KCl) and then crosslinked with 1% formaldehyde for 10 min at room temperature. The crosslinked cells were washed in ice-cold PBS, the supernatant discarded and the pellets flash-frozen in liquid nitrogen and stored at –80 °C.

**Solution hybridization capture of Hi-C library.** Hi-C samples corresponding to 750 ng were concentrated in a Speedvac then re-suspended in 3.4 μl water. Hybridization of SureSelect custom Promoter and Region Capture libraries to Hi-C libraries was carried out using Agilent SureSelectXT reagents and protocols. Post-capture amplification was carried out using six cycles of PCR from streptavidin beads in multiple parallel reactions from Hi-C libraries immobilized on Streptavidin beads, which were prepared post PCR. The final library was then sequenced on a HiSeq 2500 in 30 μl TLE and the quality and quantity assessed by Bioanalyzer and qPCR.

**Paired-end next generation sequencing.** Two biological replicates for each of the cell lines were prepared for each target capture. Sequencing was performed on Illumina HiSeq 2500 generating 75 bp paired-end reads (Genomic Technologies Core Facility in the Faculty of Life Sciences, the University of Manchester). The reads were pooled and subjected to standard bioinformatic analyses with the Hi-C User Pipeline (HCUP, http://www.bioinformatics.babraham.ac.uk/projects/hicup/). Off-target di-tags, where neither end mapped to a targeted HindIII restriction fragment, were removed from the final data sets using a combination of BEDTools and command line tools. Full details of the number and proportion of excluded di-tags are given in Supplementary Table 3.

**Analysis of Hi-C interaction peaks.** Di-tags separated by <20 kb were removed prior to analysis, as 3C data have shown a very high-interaction frequency within this distance26. Di-tags were then assigned to one of the four categories of ligations defined in the study by Dryden et al.88 using custom scripts: (1) single baited, cis interaction (<5 Mb), (2) single baited cis interactions (>5 Mb), (3) double-baited cis and trans interactions (either single or double baited). Significant interactions within 5 Mb were determined using the ‘High resolution analysis of the cis interaction peaks’ method described in the study by Dryden et al.88. To correct for experimental biases, the interactability of each fragment was determined. Interactability is calculated from the interactions from a particular baited HindIII fragment using a truncated negative binomial distribution was fitted to the distribution with the stochastic noise (low interaction) expectation for each particular baited fragment. The resulting distribution is bimodal consisting of stochastic noise (low trans counts) and genuine signal (high trans counts). A truncated negative binomial distribution was fitted to the distribution with the negative binomial truncation point for interacting restriction fragments set at a count of 3,000 and non-interacting set at 1,500 for the Promoter Capture and 600 for the Region Capture due to differences in read depth. The 5% quantile point of the non-truncated distribution was determined to provide the noise threshold. For both cell lines in both captures, the noise threshold was determined to be 400 di-tags and therefore all restriction fragments with fewer than 400 di-tags were filtered out. A negative binomial regression model was fitted to the filtered data correcting for the interactability of the captured restriction fragment and interaction distance. For
interactions, where both the target and baited region were captured (double-baited interactions), we also accounted for the interractability of the other end.

We wanted to examine whether concordance between interactions called in the Region and Promoter Capture experiments increased with decreasing FDR thresholds. This is complicated because we can only define the set of interactions that could have been observed in both experiments conditional on those that were observed at a given FDR threshold in one experiment. We therefore decided to normalize to those interactions called at an FDR threshold of 20% in the region experiment and defined the following enrichment parameter:

\[ X[i] = P \text{ (called in Region Capture at FDR } i) \text{ and in Promoter Capture at FDR } j \text{ called in Region Capture at FDR 20%)/} P \text{ (called in Region Capture at FDR } j \text{ called in Region Capture at FDR 20%).} \]

Interactions were considered statistically significant after combining replicates and filtering on FDR ≤ 5%. Significant Interactions were visualized in the WashU Epigenome Browser (http://epigenomewidget.wustl.edu/browser/).

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Author contributions

P.M., G.O., S.E. and P.F. contributed with conception and experimental design. A.M., P.M., G.O., S.E. and P.F. contributed with technical or material support. S.E., P.M., A.M., G.O., K.D., A.Y., C.W. and N.C. helped with acquisition of data. P.M., G.O., S.E. and P.F. contributed with conception and experimental design. A.M., P.M., G.O., S.E. and P.F. contributed with technical or material support. S.E., P.M., A.M., G.O., K.D., A.Y., C.W. and N.C. by the Wellcome Trust (089899); C.W. and N.C. by the Wellcome Trust (091157), IJDRF (9-2011-253) and the National Institute for Health Research (NIHR) Cambridge Biomedical Research Centre. The Cambridge Institute for Medical Research (CIMR) is in receipt of a Wellcome Trust Strategic Award (100140). The research leading to these results has received funding from the European Union’s 7th Framework Programme (FP7/2007-2013) under grant agreement no.241447 (NAIMIT) and supported by the National Institute for Health Research Manchester Musculoskeletal Biomedical Research Unit (S.E., A.B., J.W.). P.F. and S.S. were supported by Biotechnology and Biological Sciences Research Council UK grant BB/S080085/1. The views expressed in this publication are those of the authors and not necessarily those of the NHS, the National Institute for Health Research or the Department of Health.

Additional information

Accession codes: Raw data and HindIII restriction fragment interaction counts are available in the NCBI Gene Expression Omnibus (GEO); http://www.ncbi.nlm.nih.gov/geo/ under accession number GSE69600.

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