Published online 7 January 2009 *Nucleic Acids Research, 2009, Vol. 37, No. 4* ***1073–1085***
doi:10.1093/nar/gkn1051

A ChIP–chip approach reveals a novel role for transcription factor IRF1 in the DNA damage response

Mattia Frontini[1], Meeraa Vijayakumar[2], Alexander Garvin[2] and Nicole Clarke[2,*]

[1]MRC Clinical Sciences Centre, Faculty of Medicine Imperial College London, Hammersmith Hospital Campus, Du Cane Road, London, W12 0NN and [2]School of Pharmacy, Centre for Biomolecular Sciences, University of Nottingham, NG7 2RD, UK

Received November 25, 2008; Accepted December 15, 2008

ABSTRACT

IRF1 is a transcription factor that regulates key processes in the immune system and in tumour suppression. To gain further insight into IRF1's role in these processes, we searched for new target genes by performing chromatin immunoprecipitation coupled to a CpG island microarray (ChIP–chip). Using this approach we identified 202 new IRF1-binding sites with high confidence. Functional categorization of the target genes revealed a surprising cadre of new roles that can be linked to IRF1. One of the major functional categories was the DNA damage response pathway. In order to further validate our findings, we show that IRF1 can regulate the mRNA expression of a number of the DNA damage response genes in our list. In particular, we demonstrate that the mRNA and protein levels of the DNA repair protein BRIP1 [Fanconi anemia gene J (FANC J)] are upregulated after IRF1 over-expression. We also demonstrate that knockdown of IRF1 by siRNA results in loss of BRIP1 expression, abrogation of BRIP1 foci after DNA interstrand crosslink (ICL) damage and hypersensitivity to the DNA crosslinking agent, melphalan; a characteristic phenotype of FANC J cells. Taken together, our data provides a more complete understanding of the regulatory networks controlled by IRF1 and reveals a novel role for IRF1 in regulating the ICL DNA damage response.

INTRODUCTION

The interferon regulatory factor (IRF) family of proteins are important for the proper functioning and homeostasis of mammalian systems (1). The different family members play important roles in development, differentiation and immunity. In particular, IRF1 has been shown to be involved in immune responses and regulation of T-cells and myeloid cells in the immune system, cell cycle, tumour suppression and apoptosis (2–4). Most of IRF1's activity results from its binding to genes involved in these pathways and regulation of their expression. DNA-binding studies demonstrated that IRF family members bind to a consensus sequence, termed IRF-E which is very similar to the ISRE (interferon stimulated response element) found in many interferon regulated genes (5).

IRF1 is induced in response to a number of stimuli including, IFN-γ, retinoids, TNFα, bacterial infection and anti-estrogens. Gene knock out studies in mice identified IRF1 as an important immune cell regulator. $IRF1^{-/-}$ mice have aberrant lymphocyte development and when challenged with specific bacteria mount a type 2 T-helper cell response, with a marked absence of IFN-γ-producing type 1 T cells (6,7). This is thought to occur due to the absence of IL-12 and IL-18 in IRF1 null mice. IRF1 has also been shown to control positive and negative selection of $CD8^+$ thymocytes (8). The authors showed that there was an intrinsic defect in $IRF1^{-/-}$ thymocytes suggesting that IRF1 is required for lineage commitment and selection of $CD8^+$ thymocytes. IRF1 is also essential for natural killer (NK) cell function *in vivo* and in the microenvironment supporting NK cell development (9,10). IRF1 can affect tumour susceptibility in mice and harbours tumour suppressor activity (11). Different cancerous lesions undergo loss of IRF1 expression by chromosome deletion, exon-skipping and functionally inactivating point mutation (12,13). It has also been demonstrated that both IRF1 and p53 are required to prevent oncogene-induced cell transformation (14) and IRF1 can reverse the transformed phenotype both *in vitro* and *in vivo* (15).

*To whom correspondence should be addressed. Tel: +44 115 951 5058; Fax: +44 115 846 8877; Email: nicole.clarke@nottingham.ac.uk

Key to our understanding of IRF1's role in the immune system and in cancer will be the identification of direct target genes. To date, there are still relatively few gene targets mapped for IRF1. The binding of a transcription factor to the regulatory region of a specific gene suggests that the factor will have some regulatory effect on that gene. Therefore, to gain a deeper insight into IRF1-mediated regulatory networks we have undertaken a ChIP–chip study to locate IRF1-binding sites in the human genome. Using this approach, we have identified 202 new loci bound by IRF1 after IFN stimulation. We validated several of these targets by ChIP in two different cell lines and by RT–PCR of IFN-γ treated or IRF1 over-expressing cells. We observed a high ChIP validation rate (>90%) and differential transcript regulation by IFN or IRF1. Importantly, novel functions for IRF1 have emerged from this study and newly identified IRF1 bound genes can help to explain the phenotypes observed in IRF1 knockout mice.

Interestingly, a large cohort of the target genes fell under the DNA damage response category (9%). Although, a number of studies have demonstrated a strong link between IRF1 and DNA damage repair, very little is known about the target genes regulated by IRF1 in this response. We have demonstrated that one of the target genes; BRIP1 (a Fanconi anemia gene J, FANC J) is upregulated in response to IFN and IRF1 over-expression at the mRNA and protein level. We have demonstrated at the single cell level using immunofluorescence studies that downregulation of IRF1 by siRNA results in loss of BRIP1 expression and abrogation of BRIP1 foci after DNA crosslink damage. Furthermore, we demonstrate that cells that have had IRF1 expression knocked-down by small interfering RNA show a hallmark hypersensitivity to the DNA cross-linking agent, melphalan [similar to the phenotype displayed by Fanconi anemia (FA) cells]. Significantly, these results reveal a previously unknown role for IRF1 in regulating the DNA interstrand crosslink (ICL) repair pathway.

MATERIALS AND METHODS

Cells, transfection and antibodies

H3396 breast cancer cells were grown in RPMI (Lonza) and the MRC-5 lung fibroblasts were grown in MEM-alpha (Lonza). IFN-γ and Melphalan were purchased from Sigma. Antibodies used for ChIP–chip, western blots and ChIP are rabbit polyclonal anti-human IRF1 (C-20), normal rabbit IgG (sc-2027) and anti-tubulin (Santa Cruz Biotechnology), anti-BRIP1 (Autogen Bioclear). H3396 cells were plated on a 10 cm plate the day before transfection. Cells were re-fed prior to transfection (Lipofectamine, Stratagene) with 3 μg each of pCDNA-IRF1 or pCDNA 3.1 empty vector control. Twenty-four hours after transfection, the cells were lysed as per the manufacturer's protocol (Invitrogen) and RNA extracted for RT-PCR analysis.

RNA extraction and RT-PCR

RNA was extracted from H3396 cells using the RNA-EASy kit (Qiagen). For cDNA synthesis, 3 μg of total RNA was reverse transcribed using the Superscript II kit (Invitrogen). Then 1/100th of each sample was used in semi-quantitative PCR using gene-specific primers. Real time PCR was performed on the MX3005 real-time PCR (Stratagene). Primer sequences are available upon request.

ChIP

ChIP experiments were carried out as described in (43), with the exception that the Real-time PCR machine used was the MX3005P (Stratagene). The locations of the primer pairs used in the PCR reaction are depicted in Supplementary Figure 2. Primer sequences are available upon request.

Generation of ChIP amplicons for ChIP–chip

DNA amplicons for ChIP–chip were generated from three independent ChIP samples (IRF1 or IgG). Briefly, ChIP samples from each experiment (done in duplicate) were pooled and amplicons generated by LM-PCR following the protocol described (16). Briefly, three rounds of amplification were performed on T4 blunted ChIP samples to which two unidirectional linkers were annealed. Reactions were purified using the Qiagen PCR cleanup kit (Qiagen). After purification, the DNA amplicons were quantified and examined on a 2% agarose gel for size. Gene-specific PCRs were then carried out to ensure that the initial enrichment of control test genes was maintained. The average size of the amplicons was approximately 300 bp (ranging from 200 to 500 bp). Input DNA was also subjected to LM-PCR and input amplicons were then used as a reference for hybridization to a 12K CpG island (CGI) array.

Labelling, hybridization and analysis of the 12K CPG array

The 12K Human CPG microarray was purchased from the University Health Network (Toronto, Canada). DNA amplicon samples were labelled according to the protocol as described in (16). The hybridized arrays were scanned using a ScanArray 4000 (Perkin-Elmer, USA) and analysed using the Imagene 5.0 analysis software (BioDiscovery, Inc., USA). Spots that did not meet the quality control parameters after visual inspection or by the analysis software were discarded. Raw median values were normalized using the software ChiPper. Data is returned ranked according to increasing p-value. For each CpG clone, the ranked *P*-value is from two datasets for each antibody (anti-IRF1 or normal rabbit IgG). Target loci were then identified by a *P*-value cut-off of 0.001. Visualization and manual assignment of 'target' genes within 100kb of regions corresponding to the CpG clone was performed using the UHN CpG microarray database (http://data.microarrays.ca/cpg/). Functional classification of genes was carried out using the DAVID Functional annotation tool (http://david.abcc.ncifcrf.gov/). The background list used in the analysis was

generated from all the non-redundant genes within 100 kb of clones on the CGI array.

Flow cytometry

Cells were treated for 1 h with vehicle or 1 μM Melphalan 16 h after transfection with 25 nM IRF1 siRNA or control siRNA (Dharmacon-On Target Smartpool). Cells were then washed and re-fed with medium containing 10% Fetal Calf Serum and left to recover for 48 h. Cells were collected, washed 2× in PBS and fixed in 70% ethanol solution overnight. The next day cells were collected, washed in PBS and treated with RNAse A for 30 min at room temperature and then stained with propidium iodide (50 μg/ml). Cell cycle analysis was performed on a Becton Dickinson FACSARIA SORP using the DIVA software. Further analysis was performed using the program WINMDI.

Immunoflourescence

We carried out immunofluorescence microscopy studies on cells grown on coverslips. Cells were fixed and permeabilized as described (16). Coverslips were then washed in PBS and incubated in 1% PBS/BSA for 30 min. Afterwards, the coverslips were washed again in PBS and incubated overnight (4°C) in primary antibody, anti-human BRIP1 (1:300) (Santa Cruz Biotechnology). The next day, coverslips were washed in PBS and left for one hour in anti-mouse Alexa Fluor 488 conjugated secondary antibody (1:1000). Then coverslips were washed again in PBS and left for two hours with anti-human IRF1 antibody (1:1000) (Abcam). Coverslips were washed in PBS and then placed in anti-rabbit Alexa Fluor 543 conjugated secondary antibody (1:1000). All secondary antibodies were purchased from Molecular Probes, Invitrogen. A final wash in PBS was carried out and then coverslips were mounted on a glass slide in anti-fade solution (Vector Laboratories Ltd.). Images were acquired on a Zeiss LSM510 Meta laser scanning microscope.

RESULTS

Identification of loci bound by IRF1

To identify novel target genes of IRF1, we performed ChIP–chip experiments on breast cancer cells (H3396). First, we examined the expression of IRF1 after IFN-γ treatment of cells. We observed the expected increase in IRF1 after stimulation with maximal expression of IRF1 after 3 h IFN-γ treatment (Figure 1A). This time point was then chosen to perform the genome wide ChIP studies on the 12K CGI array. Cross-linked chromatin was immunoprecipitated with a polyclonal antibody against IRF1 and a normal rabbit (control) IgG antibody was used as a negative control. Figure 1B shows the enrichment of IRF1 at two known targets (TRAIL and caspase 8).

The 12K array contains genomic fragments from human CGI (17). We chose this array since (i) CGIs are known to be located in regulatory regions of genes (18,19) and there is a strong correlation between CGIs and

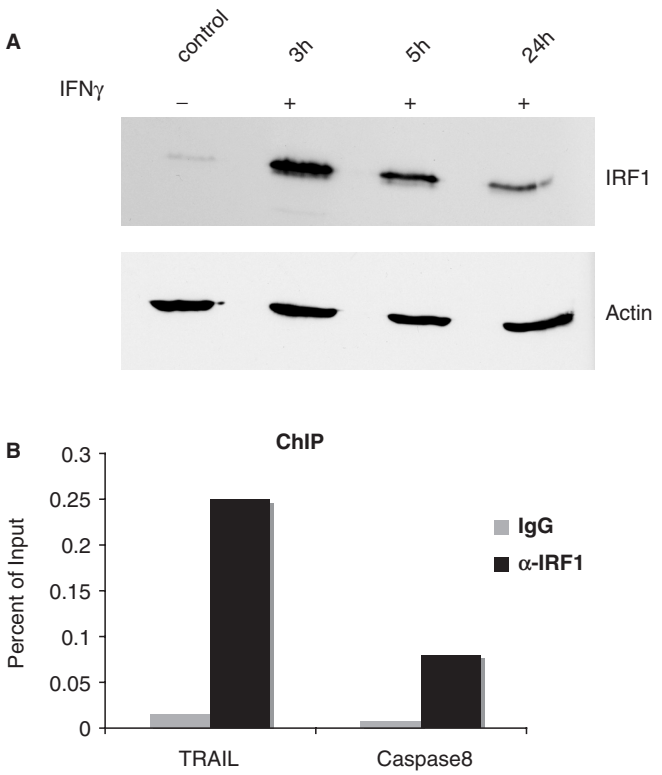

Figure 1. Western blot of IRF1 expression and control ChIP. (**A**) H3396 cells were left untreated (control) or treated with IFN-γ for the time points indicated. Shown is a western blot analysis of whole cell extracts made at each time point using an antibody against IRF1 or actin as loading control (**B**) Shown is quantitative real-time PCR of a ChIP sample from formaldehyde-fixed H3396 cells used for ChIP–chip. Cells were untreated (control) or treated with IFN-γ for 3 h and immunoprecipitated with control IgG or anti-IRF1 antibody. Two positive control genes are shown, caspase 8 and TRAIL.

clusters of transcription-factor-binding sites (20), (ii) it allowed us to screen a large number of loci in the human genome for IRF1 binding. We performed three hybridizations using independent ChIP generated amplicons. The raw ChIP–chip fluorescence intensity data were processed using ChIPper (21), a data analysis system that normalizes and scores microarray data generating *P*-values for each clone. We next eliminated redundant clones and those not mapping to known locations in the human genome. As a further control, we also generated a list of ranked clones from the IgG amplicon hybridizations. There was a very limited overlap in the two lists after the cut-off and the few IgG enriched clones that had similar p-values were removed from the IRF1 list of positive clones. Using a stringent cut-off *P*-value <0.001 and based on their location within 100 kb of transcription units (according to the hg18 NCBI build), we assigned the remaining clones to 202 genomic loci. The list of the genes, with the chromosomal position (of the CGI clone), as well as *P*-values, can be found in Supplementary Data 1.

Validation of targets

We next sought to verify that the genomic loci we identified by ChIP–chip are indeed bound by IRF1. H3396 cells

were left untreated or treated with IFN-γ for 3 h and DNA prepared from three independent ChIP experiments. Quantitative PCR was performed on 21 loci from our list of IRF-enriched genes. These loci were chosen from across the range of *P*-values in order to determine the stringency of our cut-off. To design primer pairs, we examined 1 kb upstream or downstream of the CGI clone for IRFEs or IRF-binding elements. The consensus binding sites for IRFs are well established referred to as IRFEs or ISREs (5). Almost all regions in the vicinity of the clones contained consensus IRFEs (Supplementary Data 1). We selected primers flanking potential IRF1-binding sites due to the suitability for generating single PCR fragments from the human genome. We also selected a pair of primers from the UNG gene as controls. Supplementary Figure 2 shows a schematic of the loci chosen for validation with the position of the CGI clone, consensus IRFEs (for a list of the IRFEs identified at each loci, see Supplementary Figure 6) and the primer pairs used for qPCR.

Figure 2 shows the results of the validation. Seven loci were strongly enriched for IRF1 binding after IFN treatment. These were OAS3, CCNT2, NFATC2IP, ACP2, PSMA6, C14orf43 and C6orf170. Most of these genes with the exception of NFATC2IP (*P*-value = 0.00041) ranked at the top of our enriched list according to *P*-value. Significantly, the top ranked gene, OAS3 has been shown previously to contain consensus IRFE/ISREs in its regulatory regions. This suggested that it was a target for IFN regulatory factors (22) which has now been confirmed by our study. The remaining 14 genes validated were also significantly enriched for IRF1 after IFN-γ treatment. The level of IRF1 enrichment in this group of genes also correlated well with their position in our ranked list according to p-value. It is notable that major histocompatability genes (23), as well as histone genes, (24) are regulated by IRFs. Primers to the UNG gene that were used as a negative control gave no enrichment for IRF1 over the background IgG ChIP (Figure 2). Two loci (C3orf26 and YWHAB) chosen from our target list for qPCR did not show significant enrichment for IRF1 in H3396 cells (data not shown). We also observed that a known gene target of IRF1, c-myc (1) was identified in our cohort of genes as relatively enriched for IRF1 binding (*P*-value = 0.0013) but fell below our cutoff *P*-value. This further validates our findings and suggests that other true IRF1 target genes might fall below our threshold, indicating that our cutoff value has been stringent. Overall, we obtained a validation rate of >90% (19/21), indicating that the list comprises a large number of bona fide IRF1-bound genes.

Binding of IRF1 in primary human lung fibroblast cells

We next wanted to determine if the loci tested for IRF1 binding in breast cancer cells would be bound by IRF1 after IFN treatment in another cell line. We chose the MRC5 primary lung fibroblast cell line, as this represented a normal diploid cellular environment. We first determined that IRF1 was induced in these cells after IFN-γ induction (Supplementary Figure 3A). Again, we observed an up-regulation of IRF1 protein levels after IFN induction that was maintained up to 24 h. For consistency, we chose to use the 3 h time point to perform the ChIPs. As shown in Supplementary Figure 4, all the genes tested showed similar enrichments (slightly higher in a few cases) for IRF1 after IFN treatment. Again, the UNG primers showed no enrichment for IRF1 after IFN treatment. Overall, IRF1 is bound to the same genes after IFN stimulation in both cell types and this indicates that these genes are bona fide targets of IRF1 after IFN stimulation.

We also assayed the binding of IRF1 to the ChIP–chip enriched genes in the absence of IFN (untreated cells). In both cell types (Figure 1 and Supplementary Figure 3A) we observed that IRF1 is constitutively expressed at a low level in the absence of IFN. Half of the genes displayed enrichment for IRF1 in control (non-IFN stimulated) cells compared to the background enrichment with the control IgG antibody (Figure 2 dark grey columns and Supplementary Figure 4 light blue columns). This indicates that IRF1 is bound to some of its targets prior to IFN stimulation of cells and it may play a role in their regulation in cycling cells.

Functional categories of target genes

An important aspect of this high-throughput approach is that we can assign new role(s) for IRF1 in different biological processes by looking for statistically significant functional categories in our list of genes. In order to do this, we functionally classified the ChIP–chip enriched genes into Gene Ontology (GO) categories. We performed this analysis using the DAVID functional annotation tool. Functional categorization revealed a diverse set of GO biological processes that were statistically significant in our list of genes compared to the background list, *P*-value < 0.10. Enriched categories included DNA metabolism, antigen processing, protein transport, response to DNA damage and transcription. Strikingly, we found that 8 out of the 92 genes (~9%) on the CGI array mapping to the GO DNA damage category were bound by IRF1 in our study (Figure 3 and Supplementary Figure 5). It is known that IRF1 is involved in the response of cells to DNA damage such as γ-irradiation (25,26) and may play a role in DNA repair processes (27). However, the full nature of its involvement and/or which IRF1 target genes might be involved in this process was not known. Moreover, it is known that IRF1 expression is stabilized in cells treated with DNA damaging agents (28) resulting in elevated levels of IRF1 similar to induction by IFN. Taken together the data support the idea that IRF1 plays a significant role in DNA damage/repair pathways via direct transcriptional regulation of genes involved in these processes.

As a number of the genes were not assigned to GO categories by DAVID, we sought to further categorize the genes by combining GENE Ontology data with literature supporting specific roles for the genes in the NCBI database. We manually assigned genes into GO biological processes revealing a number of novel functions. These included translation, chromosome organization and

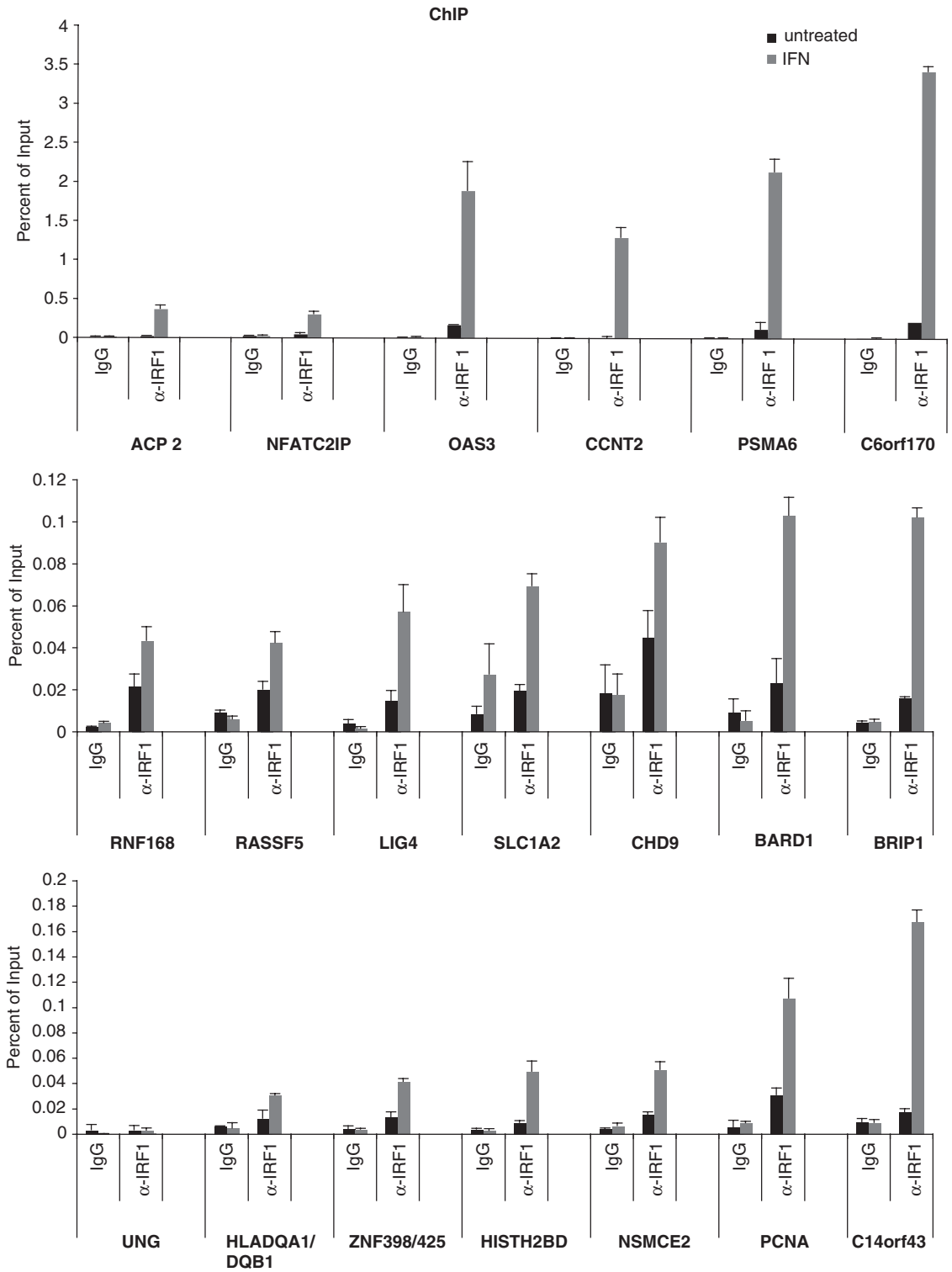

Figure 2. ChIP validation of targets. H3396 cells were untreated (dark grey bars) or treated with IFN-γ for 3 h (light grey bars) to induce IRF1 expression. ChIP was performed with control IgG or anti-IRF1 antibody. Shown is real-time PCR of immunoprecipitated DNA for IRF1 gene targets identified on the CGI array. Data are from three independent ChIP experiments ±SD. The data is displayed as Percent of INPUT chromatin.

biogenesis, signal transduction and cell adhesion. Figure 3 is a summary of this functional categorization. We identified a large number of genes involved in the immune/inflammatory or defence response. Importantly, these novel immune regulatory genes have not previously been linked to IRF1 and may be very important in understanding why IRF1 knockout mice display immune dysfunction (29).

GO Term Name	CpG Island Array	Experimental Set (IRF1 Positive)	Percent of IRF1 positive clones mapped to a Go Term from the CpG array	P-value (<0.10)
DNA metabolism	143	15	10.5%	3.00e-2
Response to DNA damage stimulis	92	8	8.7%	3.10e-2
Antigen processing	14	3	21.4%	5.70e-2
Protein transport	213	13	6.1%	6.90e-2
Transcription	912	38	4.2%	9.00e-2

Figure 3. GO functional enrichment. The table shows the statistical enrichment of GO categories assigned to the target gene list with *P*-value <0.10. Enrichment was calculated using the non-redundant list of genes on the CGI array as the background set (from the UHN CGI Microarray database) and the IRF1 target gene list (see Supplementary Figure 3). Statistical analysis was performed using the DAVID software. From right to left; column 1—GO term name from DAVID; column 2—number of genes on the CGI array mapping to the GO term; column 3—number of IRF1 bound genes mapping to the GO term; column 4—percentage of GO-mapped genes bound by IRF1 (column 3/column 2); column 5—statistical significance (*P*-value).

Expression of IRF1 bound genes

IRF1 is a direct IFN response gene and is upregulated by both type I and type II IFNs, where IFN-γ is a strong inducer of its expression (Figure 1A). As a positive control, we have determined the mRNA level of IRF1 after IFN stimulation (5 h and 24 h time points) using semi-quantitive RT-PCR (Figure 4A). As expected, we observed a large induction of IRF1 mRNA, 40–45-fold after IFN stimulation. Next, we determined the relative steady state mRNA levels of selected loci from our list of IRF1 enriched genes. In most cases, we selected the same genes as those used in Figure 2 in order to correlate expression to binding/recruitment. A number of transcripts showed transient up-regulation after IFN stimulation. These are LIG4, C6orf170 and PCNA. Other transcripts were up-regulated and remained up such as, BRIP1 (FANC J) CTDSPL2, NSMCE2 and PSMA6. Interestingly, the level of some of the transcripts did not change significantly at the time points tested after IFN stimulation. This phenomenon, where a transcription factor is recruited or bound at specific genes without any apparent consequences on the level of transcription, has been increasingly observed in different ChIP–chip studies (30–32). IRF1 can also repress transcription at a number of genes such CDK2 and CYCLIN D1, although we did not observe this effect on the set of genes we tested so far. Thus, IRF1 recruitment/binding seems to have variable effects on the level of transcription at a specific locus but it cannot be ruled out that other transcription factors also need to be recruited at a gene for a specific response to IFN. It may also be that other signals must be integrated in order to produce a change in the level of transcription.

In order to have a more direct determination of IRF1's effect on a specific locus, we over-expressed IRF1 by transient transfection (Figure 4B) and determined the level of endogenous transcripts by semi-quantitative PCR. We observed significant changes in the transcript level of six IRF1-bound genes after over-expression of IRF1 in H3396 cells, which correlated with the changes we observed after IFN treatment. Among the genes tested were four genes from the DNA damage functional category, BRIP1, PCNA, LIG4 and NSMCE2. These results suggest that IRF1 is a major player in the regulation of these genes by IFN-γ or other signals resulting in IRF1 recruitment to these genes. As a control, the level of expression of the TRAIL mRNA (a known target of IRF1) was determined. As expected there was a large increase in the level of TRAIL transcript after expression of the IRF1 cDNA.

IRF1 over-expression or IFN-γ can upregulate expression of BRIP1

One of the major functional categories identified in our ChIP–chip study was DNA damage and repair. Among these genes is BRIP1 that has a high ranking in our list of genes, *P*-value 0.000076. We found that expression of this gene is significantly upregulated at the mRNA level after both, IFN treatment and IRF1 overexpression in breast cancer cells (Figure 4A and B). These results coupled to the fact that this gene could be an unknown link between IRF1 and DNA damage, led us to investigate the regulation of BRIP1 by IRF1 in more detail. We wanted to determine if overexpression of IRF1 in cells would lead to an upregulation of BRIP1 protein similiar to what we observed at the mRNA level. Indeed, transfection of MRC5 fibroblast cells with increasing amounts of pCDN3A-IRF1 resulted in a concomitant increase in BRIP1 protein levels (Supplementary Figure 3B).

IRF1 knockdown abrogates ICL damage inducible BRIP1 foci

The protein BRIP1 is a member of the FA family of proteins, known as FANC J (33). These proteins are involved in the repair of ICLs in DNA and BRIP1 is known to form foci at DNA damage sites in response to ICL reagents (34). We reasoned that if IRF1 were involved in regulating BRIP1 expression in cells, which depletion of IRF1 by small interfering RNA (siRNA) would result in deregulation of BRIP1 expression or foci formation. Therefore, we performed immunofluorescence experiments and confocal microscopy to look at individual cells and the response to melphalan after IRF1 knockdown. We determined the levels of BRIP1 and IRF1 at the single cell level, before and after ICL damage. In cells transfected with control siRNA, we observed a clear nuclear staining for IRF1 (Figure 5A and B), which is consistent with other studies showing IRF1 as a mainly nuclear protein (35). In control cells, BRIP1 also displayed nuclear staining (Figure 5A). We observed no co-localization of these two proteins (see merged, Figure 5). After control cells were treated with melphalan (Figure 5B), we observed a large increase in BRIP1 foci. In contrast, cells transfected with IRF1 siRNA, displayed very weak residual BRIP1 staining (Figure 5C) and no staining for IRF1. After melphalan treatment of IRF1

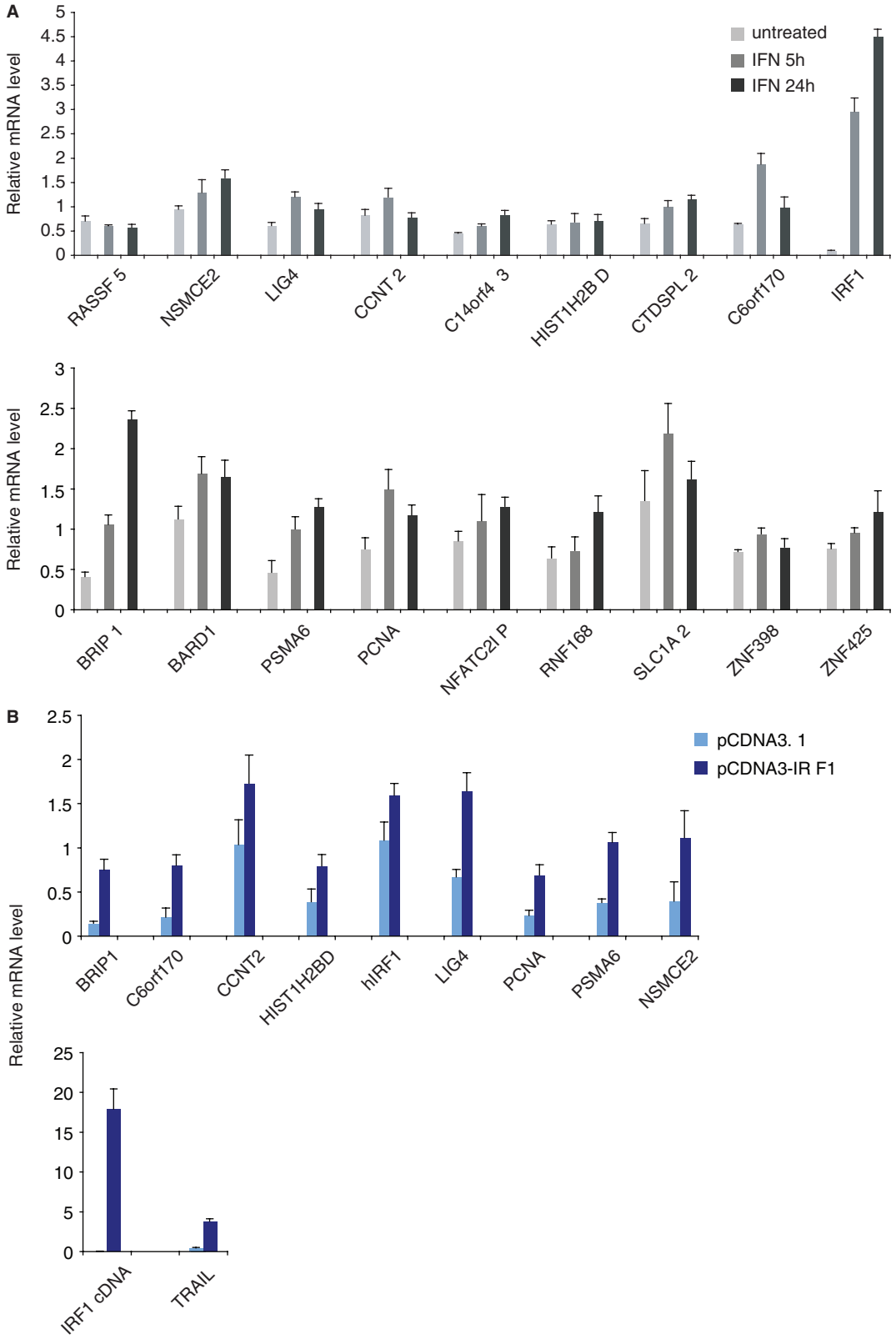

Figure 4. Expression analysis of target genes. (**A**) H3396 cells were untreated or treated with IFN-γ for 5 and 24 h. Cells were collected and lysed for RNA extraction. RNA was used for RT–PCR and real-time PCR analysis, using gene-specific primers. Data shown represents three independent samples ±SD. All data are normalized to β-actin mRNA (**B**) H3396 cells were transfected with 5 μg of empty vector (pCDNA3) or pCDNA3-IRF1. Cells were collected 24 h later and lysed for RNA extraction. RNA was used for RT–PCR and real-time PCR analysis. Data shown are from three independent transfections ±SD. All data are normalized to β-actin mRNA.

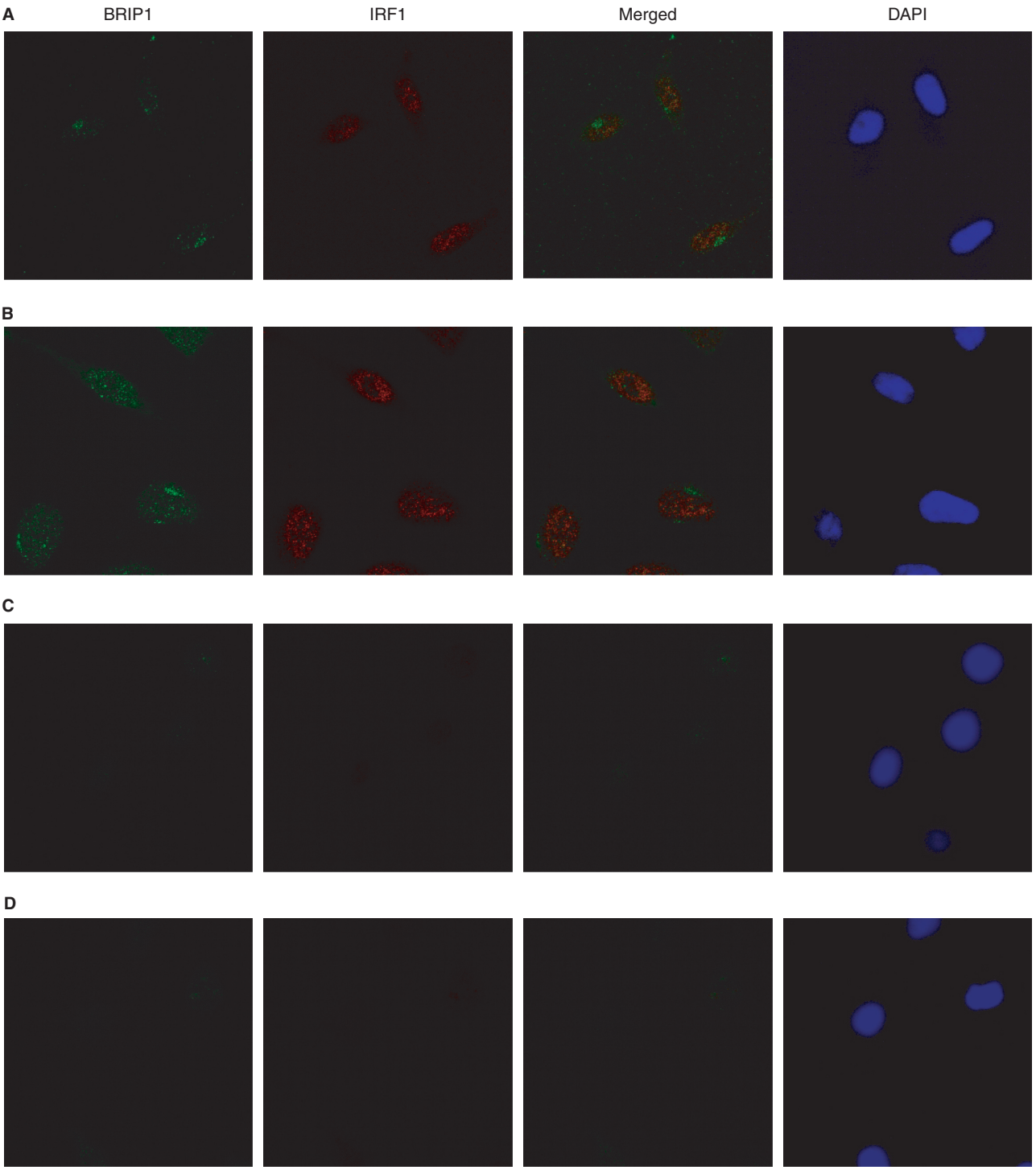

Figure 5. IRF1 expression is required for BRIP1 expression and foci formation after ICL damage. MRC5 cells transfected with 50 nM of control (**A, B**) or IRF1 siRNA (**C, D**) were treated with 500 nM melphalan (B, D) for 16 hrs and stained with either IRF1 or BRIP1 antibody as indicated and with DAPI.

knockdown cells, we observed no DNA damage induced BRIP1 foci (compare BRIP1 in Figure 5D to B). These results demonstrate that IRF1 is involved in regulating the expression of the BRIP1 protein in undamaged cells and that BRIP1 foci formation after ICL (melphalan) damage is dependent on IRF1 expression. The fact that we observed a very low level residual BRIP1 staining in untreated cells (that did not change after melphalan treatment), argues that there are other factors that may also be responsible for regulating expression of BRIP1 in undamaged, cycling cells such as E2F1, which has recently been shown to regulate its expression (36).

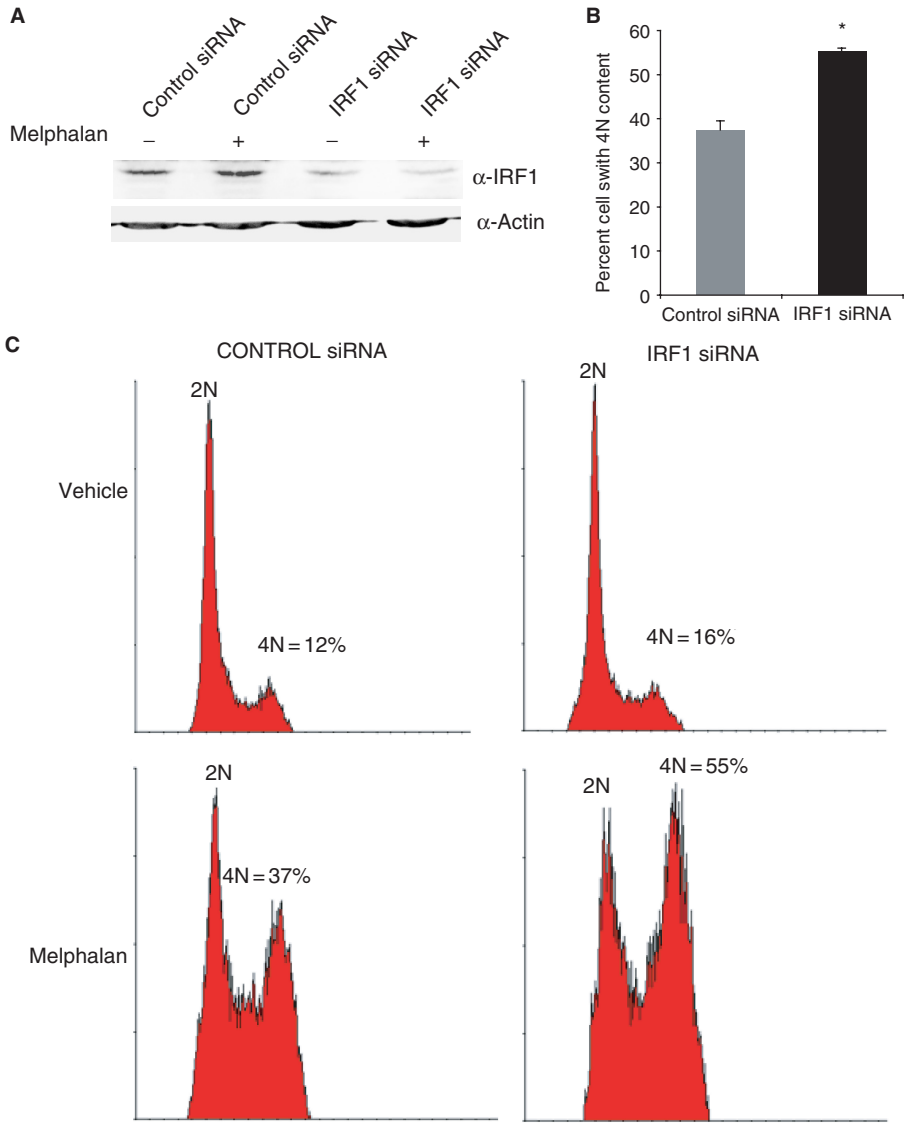

Figure 6. Knockdown of IRF1 results in hypersensitivity to melphalan with increased accumulation of cells in G2/M. (**A**) MRC5 cells were transfected with 25 nM of control siRNA or IRF1 siRNA and treated with vehicle or melphalan (1 μM) for 1 h. Western blot analysis shows IRF1 protein levels in control and IRF1 siRNA transfected cells after 64 h. β-actin is shown as a control. (**B**) Cell cycle analysis was performed on the above cells. Shown is a graph depicting the percent of cells with 4N (G2/M) DNA content as measured by propidium iodide uptake, Student *t*-test and $^{*}P$-value is 0.007. Data shown are the mean of three experiments ±SD. (**C**) A representative experiment is shown from the flow cytometric analysis of cells transfected with control or IRF1 siRNA for 16 h and then treated with melphalan (1 μM) for 1 h. Cells were collected and stained with propidium iodide 64 h after transfection.

IRF1 knockdown increases sensitivity of cells to ICL damage

Our immunofluorescence data then prompted us to probe whether IRF1's regulation of BRIP1 could link IRF1 to the ICL repair pathway. Therefore, we next determined if downregulation of IRF1 could affect the sensitivity of cells to ICL inducing agents. It is known that treatment of FA cells with DNA crosslink reagents such as mitomycin C or melphalan results in a marked hypersensitivity with increased accumulation of cells in G2/M or with near 4N DNA content (37,38). In order to test this, we decreased IRF1 protein levels using IRF1 siRNA (Figure 6A) and determined the DNA content of cells by cell cycle analysis using propidium iodide. This experiment was performed in cells before and after treatment with melphalan. Interestingly, we observed an increased hypersensitivity to melphalan in cells with decreased levels of IRF1 protein. Cells with IRF1 siRNA had a marked increase in 4 N DNA content compared to cells transfected with the control siRNA (Figure 6B and C). These results demonstrate that a deficiency in IRF1 leads to a defect in ICL repair and hypersensitivity to crosslinking agents.

DISCUSSION

IRF1 is a transcription factor controlling anti-cancer, apoptotic and immune/inflammatory responses. To understand the role of IRF1 in these responses, the

identification of its transcriptional targets is important. Therefore, we performed a ChIP–chip analysis to identify IRF1-binding sites in the human genome. Using this approach, we were able to identify 202 novel loci that are enriched for IRF1 binding after IFN treatment of cells. The validation experiments confirmed that >90% of these loci are bound by IRF1 *in vivo*, proving the effectiveness of our approach. Correlation of ChIP-validated genes with expression analysis, after IFN treatment or over-expression of IRF1, showed that at least half of the bound genes were regulated after IRF1 recruitment/binding. We also identified consensus IRFEs or ISREs in the close vicinity of most of the CGI clones. These results increase our confidence in the ranking and identification of IRF1 bound genes.

Functional characterization of the bound genes revealed that IRF1 potentially controls many diverse processes in mammalian systems. Importantly, most of the bound genes identified have not been previously linked to IRF1. Our results suggest that IRF1 is involved in regulating genes that encode components of the DNA damage response and DNA repair pathways, as well as factors involved in chromatin biogenesis and organization, cell cycle processes, transport (protein and vesicle-mediated), immune and inflammatory responses, and transcription.

IRF1 tumour suppressor activity may be linked to new targets identified by ChIP–chip

We have identified new IRF1 bound genes that may play a critical role in its tumour suppressor activity (11). Some of IRF1's tumour suppressor activity is linked to its immune regulatory functions (39–41) but it also has cell intrinsic anti-cancer activity that has not been fully characterized. IRF1 is involved in a range of processes affecting cancer formation such as DNA damage responses, cell cycle and apoptosis but very few genes in these categories have actually been defined as IRF1 target genes. IRF1 is well known to induce two apoptotic factors, TRAIL and caspase 8 (42,43) and the cell cycle inhibitor p21 is induced by IRF1 in cooperation with the tumour suppressor p53 (25). Recently, the tumour suppressor activity of IRF1 has also been linked to the downregulation of cyclin D1 (44) and survivin (45).

Supplementary Figure 7 lists the IRF1 bound genes that have been shown to harbour anti-proliferative or tumour suppressor activity. Two of these genes, BRIP1 and BARD1, are important proteins that interact with the breast cancer tumour suppressor gene, BRCA1 (46,47). IRF1 is able to inhibit the growth of mammary tumour cells *in vitro* and *in vivo*, in mouse tumour xenograft models (48) and is involved in the repression of mammary tumour cells after treatment with tamoxifen (49). Also, IRF1 expression negatively correlates with breast tumour grade, where high-grade tumours were less likely to maintain expression of IRF1 (50), suggesting that IRF1 expression may be antagonistic to breast cancer progression. The fact that two genes important in the maintenance of genomic integrity in mammary cells have been found in our list of target genes reinforces this idea. Five other target genes, HPGD, PLAGL1, RASSF5, AKAP12 and DCC have been reported to harbour tumour suppressor activity. (51–61). It will be of interest in future studies to identify the role of these individual genes in IRF1's anti-cancer activity.

New functional role for IRF1 in regulating DNA interstrand crosslink repair

In addition to the above genes, novel IRF1 bound genes identified included a large subset of genes involved in DNA damage responses and DNA repair. Here, we have provided evidence that IRF1 plays a previously unknown role in regulating ICL repair. We have shown that IFN-γ that highly induces IRF1 expression results in increased IRF1 binding at the promoter region of the BRIP1 gene. This binding results in gene activation since over-expression of IRF1 leads to upregulation of BRIP1 mRNA and protein. BRIP1 was initially identified as an interacting partner of BRCA1 (62) and is a member of the FA protein family (63). FA is a multigene disease, characterized by bone marrow failure and developmental abnormalities. These proteins have been shown to play essential roles in DNA repair and owing to this, FA cells have a high degree of genomic instability and cancer predisposition (33,64–67). In particular, the FA pathway has been shown to play an important role in DNA ICL repair, where FA cells display a hallmark 'hypersensitivity' to DNA cross-linking agents. There are 13 family members to date; with BRIP1 being the FA protein J that forms part of the Group III FA proteins which function downstream of the Group II FA proteins (FANCD2 and FANCI) in DNA repair (for a more comprehensive discussion, please see Wang, 2007 and references within). The exact role that BRIP1 plays has not been fully elucidated but it has been hypothesized to facilitate homologous recombination or translesional bypass at stalled replication forks (33,63).

Given BRIP1's important role in the FA pathway, we wanted to test if IRF1 might then be involved in regulating DNA damage responses to ICLs. Indeed, our data supports an active role for IRF1 in regulating this repair pathway, as cells with diminished IRF1 expression are hypersensitive to the DNA cross-linking agent, melphalan. It is important to note that this hypersensitivity is a peculiarity of FA null cells or cells with defects in other proteins that have been shown to affect the ICL repair pathway (68,69). Thus, these data definitively link IRF1 to repair processes required for resolution of ICLs. Importantly, we could not detect appreciable BRIP1 expression in IRF1 knockdown cells. We also show that in the absence of IRF1 expression there is an abrogation of BRIP1 foci in ICL damaged cells. We conclude from these results that IRF1 is important for maintaining levels of BRIP1 in cells and subsequently for the formation of sufficient BRIP1 foci after ICL damage. The known fact that loss of BRIP1 expression in cells results in hypersensitivity to cross-linking agents (63,70) suggests that the major defect contributing to the ICL hypersensitivity we observe in IRF1 knockdown cells is the abrogation of BRIP1 expression. Although, we cannot rule out that other DNA repair or DNA damage response genes

regulated by IRF1 are also involved in the ICL hypersensitivity of IRF1 knockdown cells. Our ChIP–chip study has identified a number of other target proteins involved in DNA repair such as PCNA. It will be of interest in the future to determine the extent to which IRF1 regulates these other DNA repair proteins and whether they are also involved in DNA damage responses where IRF1 is involved.

In summary, we have successfully identified several bona fide IRF1 bound genes, before and after IFN stimulation in two cell types. Since IRF1 was first identified, many studies have elucidated its' role in the immune system and in tumour suppression. We have been successful in greatly strengthening these known roles by identifying many novel gene targets of IRF1 that are involved in these two processes. We have also been able to demonstrate a new function for IRF1, the regulation of DNA ICL repair. IRF1 regulates one of the components of the FA/BRCA DNA repair pathway, BRIP1. This pathway is important for maintaining genomic integrity in cells after genotoxic insult. Moreover, BRIP1 is a newly identified breast cancer susceptibility gene, with a doubling of breast cancer risk (71–73). Therefore, our data linking IRF1 to BRIP1 further demonstrates and better explains IRF1's role as a known tumour susceptibility gene (11).

ACKNOWLEDGEMENTS

We would like to thank C. Bole-Feysot and D. Dembele for help and advice on protocols and statistical analysis. We would also like to thank C. De Moor for help with confocal microscopy and fruitful discussions during preparation of this manuscript.

FUNDING

This research was supported by funds from the University of Nottingham, BBSRC and Cancer Research, UK. Funding for open access charge: BBSRC.

Conflict of interest statement. None declared.

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