HCFC2 is needed for IRF1- and IRF2-dependent Tlr3 transcription and for survival during viral infections

Lei Sun,1 Zhengfan Jiang,3 Victoria A. Acosta-Rodriguez,2 Michael Berger,3 Xin Du,3 Jin Huk Choi,1 Jinhui Wang,1 Kuan-wen Wang,1 Gokhul K. Kilaru,3 Jennifer A. Mohawk,2 Jiexia Quan,1 Lindsay Scott,4 Sara Hildebrand,1 Xiaohong Li,1 Miao Tang,1 Xiaoming Zhan,1 Anne R. Murray,1 Diantha La Vine,1 Eva Marie Y. Moresco,1 Joseph S. Takahashi,2 and Bruce Beutler1

1Center for the Genetics of Host Defense and 2Department of Neuroscience, Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX
3Department of Genetics, The Scripps Research Institute, La Jolla, CA

The Rockefeller University Press

Article

Transcriptional regulation of numerous interferon-regulated genes, including Toll-like receptor 3 (Tlr3), which encodes an innate immune sensor of viral double-stranded RNA, depends on the interferon regulatory factor 1 (IRF1) and IRF2 transcription factors. We detected specific abrogation of macrophage responses to polyinosinic-polycytidylic acid (poly(I:C)) resulting from three independent N-ethyl-N-nitrosourea–induced mutations in host cell factor C2 (Hcfc2). Hcfc2 mutations compromised survival during influenza virus and herpes simplex virus 1 infections. Hcfc2 promoted the binding of IRF1 and IRF2 to the Tlr3 promoter, without which inflammatory cytokine and type I IFN responses to the double-stranded RNA analogue poly(I:C) are reduced in mouse macrophages. Hcfc2 was also necessary for the transcription of a large subset of other IRF2-dependent interferon-regulated genes. Deleterious mutations of Hcfc2 may therefore increase susceptibility to diverse infectious diseases.

INTRODUCTION

A sensor of double-stranded RNA (dsRNA), TLR3 mediates host defense against numerous viruses, including HSV-1 (Zhang et al., 2007), encephalomyocarditis virus (Hardarson et al., 2006; McCartney et al., 2011), respiratory syncytial virus (Rudd et al., 2005), hepatitis B virus (Karimi-Googheri and Arababadi, 2014), and influenza A virus (IAV; Le Goffic et al., 2007; McCartney et al., 2011). Once activated, TLR3 signals via the adapter TRIF (TIR domain–containing adapter inducing IFN-β, also called TICAM-1), NF-κB, MAPKs, and IFN regulatory factor 3 (IRF3), leading to the production of proinflammatory cytokines and type I IFN (Yamamoto et al., 2002).

Tlr3 expression is itself regulated by type I and II IFNs, which drive a transcriptional program mediated by STAT and IRF transcription factors (Ivashkiv and Donlin, 2014; Schneider et al., 2014). TLR3 mRNA expression is regulated by IRF1, IRF2, and IRF8, which bind to at least one IRF element (IRF-E) in the Tlr3 promoter to positively (IRF1 and IRF2) or negatively (IRF8) affect transcription (Heinz et al., 2003; Fragale et al., 2011). Both IRF1 and IRF2 are necessary for Tlr3 transcription induced by IFN stimulation or viral infection, whereas only IRF2 is required for maintenance of the basal (unstimulated) level of Tlr3 expression (Heinz et al., 2003; Nhu et al., 2006; Ren et al., 2015). By recruiting chromatin remodeling complexes such as BAFF-R, IRF2 promotes an open chromatin state necessary for basal and induced Tlr3 transcription (Ren et al., 2015). IRF1 is a strong activator of Tlr3 transcription and is believed to compete with IRF2 for IRF-E binding after IFN stimulation (Ren et al., 2015).

At least nine IRF family members exhibit sequence and structural homology in their DNA-binding domains and therefore bind to similar IRF-E DNA sequences. The crystal structures of the DNA-binding domains of IRF1 or IRF2 in complex with a consensus IRF-E sequence showed that the IRFs induce DNA bending that may promote cooperative binding of additional IRFs and other transcription factors (Escalante et al., 1998; Fujii et al., 1999). However, little is known concerning the mechanisms by which IRFs discriminate between IRF-Es in different genes.

Using forward genetic analysis in mice, we discovered that HCFC2 is a critical component of the IRF1 and IRF2
transcriptional machinery that regulates Tlr3 and selected interferon-regulated gene (IRG) expression. The essential nature of HCFC2 is underscored by an increased susceptibility to viral infections in Hcfc2-deficient mice.

RESULTS

The feckless phenotype: Impaired TLR3 signaling

To identify genes involved in extracellular dsRNA sensing and signaling, we screened thioglycolate-elicited peritoneal macrophages (PMs) from third-generation descendants of N-ethyl-N-nitrosourea (ENU)–mutagenized C57BL/6J mice for TNF and IFN-β production in response to polyinosinic-polycytidylic acid (poly(I:C)). Short-term (4 h) treatment guaranteed that endosomal TLR3 signaling served as the major dsRNA sensory mechanism (Fig. 1 A).

A recessive phenotype with minor heterozygote effect, which we named feckless (fls) to denote a weak and ineffectual innate immune response, was characterized by reduced TNF production in response to poly(I:C) (Fig. 1 A). TNF production was normal in fls homozygous PMs in response to the TLR ligands Pam 3CSK4 (TLR2/1), macrophage-activating lipopeptide-2 (MALP-2; TLR2/6 ligand; C), LPS (TLR4 ligand; D), flagellin (TLR5 ligand; E), R848 (TLR7 ligand; F), and CpG-oligodeoxynucleotide 1668 (CpG-ODN; TLR9 ligand; G) in vitro at the indicated concentrations. TNF in the culture medium was measured by ELISA 4 h later. Tlr3−/− macrophages served as a negative control (A). *, P ≤ 0.05; ****, P ≤ 0.0001 (two-way ANOVA). Data represent mean ± SEM; n = 4 mice per genotype. (H) Immunoblot analysis of phosphorylated (p) ERK, p-JNK, p-p38, and the degradation of IκB at the indicated times after TNF treatment of PMs from fls/fls or WT mice. Total proteins and actin were used as loading controls. Results are representative of three independent experiments.

A mutation of HCFC2

Genome-wide linkage analysis was performed to identify the mutation responsible for the fls phenotype (Xia et al., 2010).
A single linkage peak on distal chromosome 10 (logarithm of odds = 7.9527) established a 48.5-Mb critical region delimited by markers at 63,084,902 and 111,639,564 bp (Fig. 2 A). Whole-exome sequencing of DNA from an affected mouse identified a missense mutation within the critical region, a T-to-C transition at base pair 82,712,061 on chromosome 10. The mutation affects base pair 1,023 of the Hcfc2 mRNA and results in a tryptophan-to-arginine substitution at amino acid 296 of HCFC2 (Fig. 2, B and C). Compound heterozygosity for the Hcfc2fls allele and either of two other ENU-induced Hcfc2 missense alleles (Fig. 2 C) reproduced the fls phenotype (Fig. 2 D). Moreover, homozygosity for a TALEN-mediated knockout allele of Hcfc2 resulted in a more severe defect than observed in fls homozygotes, suggesting that the fls allele is hypomorphic (Fig. 2 E). These data confirmed that HCFC2 is necessary for the response to poly(I:C) in macrophages.

Immunoblot analysis of lysates of bacteria expressing either WT HCFC2 or HCFC2fls revealed reduced HCFC2fls protein expression compared with WT HCFC2 (Fig. 2 F) despite comparable levels of transcript expression (Fig. 2 G), suggesting that the fls mutation causes protein instability and degradation.

**Impaired TLR3 expression caused by HCFC2 fls**

The defect of poly(I:C)-induced TNF production in Hcfc2fls/fls PMs suggested that TLR3-associated MAPK signaling, NF-kB signaling, or both might be disrupted. Homozygous fls PMs displayed reduced phosphorylation of p38, JNK, and extracellular signal–regulated kinase (ERK), and impaired IkB degradation in response to poly(I:C) treatment (Fig. 3 A), indicating that MAPK and NF-kB signaling is disrupted. Moreover, poly(I:C)-induced IFN-β production was impaired in Hcfc2fls/fls PMs (Fig. 3 B), as was phosphorylation of TBK1 (Fig. 3 A), indicating a defect of the IRF3-dependent pathway. IFN-β augments its own synthesis through an autoamplification loop involving JAK1, TYK2, and STAT1 (Darnell et al., 1994; Stark et al., 1998). In line with the observed reduction in IFN-β production, poly(I:C)-induced STAT1 phosphorylation was diminished in Hcfc2fls/fls PMs (Fig. 3 A). Thus, both the pathway leading to proinflammatory cytokine production and the pathway leading to type I IFN production were disrupted in homozygous fls PMs, indicating that TLR3 signaling was blocked either at the point of divergence of these two pathways (TRIF) or further upstream.

To test the effect of the fls mutation on TRIF function, we examined LPS-induced signaling in Myd88−/− and Myd88+/−; Hcfc2fls/fls PMs, which transduce TLR4 signals only through TRIF PMs from Myd88−/− and Myd88−/−; Hcfc2fls/fls mice displayed an equivalent magnitude and time course of ERK, p38, and STAT1 phosphorylation (Fig. 3 C), demonstrating that the fls mutation does not impair TRIF function. In addition to TRIF, TLR3 also recruits and activates phosphoinositide 3-kinase (PI3K) and its substrate, Akt, to fully activate IRF3 (Sarkar et al., 2004). Akt phosphorylation was abrogated in poly(I:C)-activated Hcfc2fls/fls PMs (Fig. 3 A).

TLR3 ligands must be brought into endosomes to interact with receptors, and the cell surface protein MSR1 facilitates uptake of poly(I:C) before delivery to endosomes (Limmon et al., 2008; DeWitte-Orr et al., 2010). Whereas TLR3 signaling in Msr1−/− PMs was rescued when the cationic liposomal transfection reagent DOTAP was used to artificially deliver poly(I:C) to endosomes, neither Tlr3−/− nor Hcfc2fls/fls PM responses were improved under the same conditions (Fig. 3 D), indicating that defective responses of Hcfc2fls/fls PMs were not due to impaired poly(I:C) uptake.

Because our data indicated a defect at the level of the TLR3 receptor caused by the HCFC2fls mutation, we examined TLR3 mRNA and protein expression levels in HCFC2-deficient cells. We found that TLR3 protein was reduced in Hcfc2fls/fls PM lysates relative to WT PM lysates; both the endosomally cleaved functional form (García-Cattaneo et al., 2012; Qi et al., 2012; Toscano et al., 2013) and the uncleaved form of TLR3 (trafficked from ER–Golgi) were affected (Fig. 3 E). We also found reduced transcript levels of Tlr3 in unstimulated Hcfc2fls/fls PM lysates (Fig. 3 F). In Hcfc2fls/fls BMDMs, both basal and IFN-β–induced Tlr3 transcription were significantly lower than in WT BMDMs, although the fold increase in Tlr3 transcripts resulting from IFN-β treatment was similar between Hcfc2fls/fls and WT BMDMs (Fig. 3 G). These findings suggest that reduced TLR3 protein expression stems from impaired TLR3 transcription in macrophages lacking HCFC2. We conclude that defective TLR3 signaling in Hcfc2fls/fls macrophages is caused by inadequate amounts of TLR3.

**HCFC2 facilitates IRF1/2 binding to the Tlr3 promoter**

HCFC1 binds to several transcription factors and chromatin modification enzymes via its β-propeller domain (Kristie et al., 1995; Wysocka and Herr, 2003), and we hypothesized that HCFC2 may bind and regulate the activity of transcription factors that control Tlr3 expression. We used mass spectrometry of immunoprecipitated FLAG–tagged HCFC2 complexes to identify its interacting partners. Known interacting proteins, including histone–lysine N-methyltransferase SETD1A, retinoblastoma–binding protein 5 (RBBP5), and WD repeat–containing protein 5 (WDR5), were recovered in the HCFC2 precipitate, validating the experiment protocols and Table 1. We also identified IRF2 among the immunoprecipitated proteins (Table 1), and we tested the possible interaction between HCFC2 and either IRF2 or its closely related family member IRF1. We found that HCFC2 and IRF2 coimmunoprecipitated from 293T or THP-1 cell lysates (Fig. 4 A and B). Moreover, HCFC2 kelch-like repeats 5 and 6 within the β-propeller domain were minimally required for interaction with IRF2, while the IRF association domain of IRF2 was needed for binding to HCFC2 (Fig. S2). An association between HCFC2 and IRF1 was also detected (Fig. 4 A).
We used gel shift experiments to test whether HCFC2 affects IRF1 or IRF2 binding to the Tlr3 IRF-E. By itself, purified HCFC2 failed to bind a 28-bp DNA probe containing the IRF-E (Fig. 4 C, second lane), whereas shifted migration of the probe indicated formation of an IRF2/IRF-E complex (Fig. 4 C, third lane). Strikingly, increased IRF2/IRF-E complex formation was observed upon addition of HCFC2 to the reaction containing IRF2 and IRF-E DNA (Fig. 4 C, fourth lane); this complex was supershifted by an IRF2 antibody but not an HCFC2 antibody (Fig. 4, F and G). HCFC2 immunoblot analysis (F) and qRT-PCR measurement of Hcfc2 mRNA normalized to Gapdh (G) in lysates of 3T3 cells stably transfected with plasmids expressing eGFP, WT HCFC2, or HCFC2fls. Data represent mean ± SEM. Results are representative of two independent experiments.
C [sixth and seventh lanes] and D), indicating that HCFC2 was not part of the IRF2/IRF-E complex. HCFC2 exerted a similar effect on the interaction between IRF1 and IRF-E DNA (Fig. 4 E). Consistent with these data, gel shift analysis performed using nuclear extracts of WT or Hcfc2−/− PMs demonstrated reduced IRF2/IRF-E complex formation by Hcfc2−/− extracts relative to WT extracts (Fig. 4 F). To provide further evidence, chromatin immunoprecipitation (ChIP) using an IRF2 antibody was performed and significantly reduced association between IRF2 and the IRF-E site in the Tlr3 promoter was found in Hcfc2−/− MEFs compared with WT MEFs (Fig. 4 G).
In summary, the data in this section suggest that HCFC2 forms complexes with IRF2 and IRF1 to facilitate their binding to the Tlr3 IRF-E and that HCFC2 releases these IRFs upon their binding to DNA.

**Genome-wide analysis of gene regulation by IRF2 and HCFC2**

IRF1 and IRF2 regulate the transcription of numerous IRGs in addition to Tlr3. To investigate whether HCFC2 may be important for IRF2 transcriptional regulation of genes other than Tlr3, ChIP sequencing (ChIP-seq) was conducted using an IRF2 antibody and either WT or Hcfc2 MEFs (Fig. 5 A). A total of 6,369 DNA-binding sites for IRF2 were identified in WT and Hcfc2−/− samples combined (Tables S1 and S2), and among them 381 were differentially enriched in either WT or Hcfc2−/− samples (Table S3); greater quantities of 365 (95.8%) DNA sequences were immunoprecipitated from WT MEFs relative to Hcfc2−/− MEFs (Fig. 5 B), whereas 16 (4.2%) sequences were increased in IRF2 immunoprecipitates from Hcfc2−/− MEFs relative to WT MEFs. Strikingly, motif analysis of the 365 binding sequences that were enriched in WT samples showed that 55.07% matched the consensus IRF2-binding site (Fig. 5 C; P = 10−227); none of the 16 Hcfc2−/−-enriched binding sequences matched the IRF2 consensus binding sequence. Analysis of the flanking regions found no significantly preferred sequence. These findings suggest that HCFC2 is necessary for the association of IRF2 with several of its targets.

As a complement to the ChIP-seq data, we performed RNA sequencing (RNA-seq) to examine the genome-wide transcriptional consequences of Hcfc2 or Irf2 deficiency in BMDMs. Pearson correlation coefficient analysis showed strong correlations between biological replicates in each group (Fig. 6 A). When normalized to WT BMDMs, Hcfc2−/− BMDMs showed significant changes of expression in 1,210 genes, and Irf2−/− showed significant changes of expression in 1,026 genes. Altogether, the expression of 571 genes was similarly altered (increased or decreased) in Hcfc2−/− and Irf2−/− BMDMs compared with WT BMDMs; these represent 47% (571 of 1,210) and 56% (571 of 1,026) of all genes affected by the respective mutations (Fig. 6, B and C). After IFN-β treatment, 71% (403 of 571) of the genes that were similarly affected by Irf2 and Hcfc2 mutations remained significantly differentially expressed, with both mutations resulting in the same direction of change relative to WT samples (Fig. 6 C).

Comparison of ChIP-seq and RNA-seq data revealed a total of 31 genes that showed both reduced association with IRF2 and altered transcript levels in Hcfc2−/− samples relative to WT MEFs.

<table>
<thead>
<tr>
<th>Protein description</th>
<th>Spectral counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNRN2_HUMAN Heterogeneous nuclear ribonucleoprotein H2 OS = Homo sapiens GN = HNRNPH2 PE = 1 SV = 1</td>
<td>94.00</td>
</tr>
<tr>
<td>SET1A_HUMAN Histone-lysine N-methyltransferase SET/DNMT3A OS = Homo sapiens GN = SETD1A PE = 1 SV = 3</td>
<td>47.08</td>
</tr>
<tr>
<td>DIO1_HUMAN Death-inducer obliterator 1 OS = Homo sapiens GN = DIO1 PE = 1 SV = 5</td>
<td>31.88</td>
</tr>
<tr>
<td>CXXC1_HUMAN CXXC-binding protein OS = Homo sapiens GN = CXXC1 PE = 1 SV = 2</td>
<td>27.78</td>
</tr>
<tr>
<td>SIRT1_HUMAN NAD-dependent protein deacetylase sirtuin-1 OS = Homo sapiens GN = SIRT1 PE = 1 SV = 1</td>
<td>25.86</td>
</tr>
<tr>
<td>WDR5_HUMAN WD repeat-containing protein 5 OS = Homo sapiens GN = WDR5 PE = 1 SV = 1</td>
<td>22.00</td>
</tr>
<tr>
<td>RBP5_HUMAN Retinoblastoma-binding protein 5 OS = Homo sapiens GN = RBPB5 PE = 1 SV = 2</td>
<td>22.00</td>
</tr>
<tr>
<td>BD1L1_HUMAN Biorientation of chromosomes in cell division protein 1-like 1 OS = Homo sapiens GN = BD1L1 PE = 1 SV = 2</td>
<td>19.98</td>
</tr>
<tr>
<td>FSHB_HUMAN Follicle-stimulating hormone beta OS = Homo sapiens GN = FSHBPE = 1 SV = 1</td>
<td>15.96</td>
</tr>
<tr>
<td>ZNG639_HUMAN Zinc finger protein 639 OS = Homo sapiens GN = ZNF639 PE = 1 SV = 1</td>
<td>12.92</td>
</tr>
<tr>
<td>THAP1_HUMAN THAP domain-containing protein 11 OS = Homo sapiens GN = THAP1 PE = 1 SV = 2</td>
<td>12.00</td>
</tr>
<tr>
<td>ZBTB2_HUMAN Zinc finger and BTB domain-containing protein 2 OS = Homo sapiens GN = ZBTB2 PE = 1 SV = 1</td>
<td>11.00</td>
</tr>
<tr>
<td>NUCC3_HUMAN NudC domain-containing protein 3 OS = Homo sapiens GN = NUCCD3 PE = 1 SV = 3</td>
<td>10.00</td>
</tr>
<tr>
<td>THAP7_HUMAN THAP domain-containing protein 7 OS = Homo sapiens GN = THAP7 PE = 1 SV = 2</td>
<td>5.00</td>
</tr>
<tr>
<td>F8W9N3_HUMAN Ubiquitin carboxyl-terminal hydrolase BAP1 OS = Homo sapiens GN = BAP1 PE = 2 SV = 1</td>
<td>5.00</td>
</tr>
<tr>
<td>KDM4A_HUMAN Lysine-specific demethylase 4A OS = Homo sapiens GN = KDM4A PE = 1 SV = 2</td>
<td>4.00</td>
</tr>
<tr>
<td>THAP9_HUMAN DNA transposase THAP9 OS = Homo sapiens GN = THAP9 PE = 1 SV = 2</td>
<td>4.00</td>
</tr>
<tr>
<td>BACH1_HUMAN Transcription regulator protein BACH1 OS = Homo sapiens GN = BACH1 PE = 1 SV = 2</td>
<td>4.00</td>
</tr>
<tr>
<td>SHCIT1_HUMAN Shoc1 OS = Homo sapiens GN = RIA1598 PE = 1 SV = 4</td>
<td>4.00</td>
</tr>
<tr>
<td>KMT2A_HUMAN Histone-lysine N-methyltransferase 2A OS = Homo sapiens GN = KMT2A PE = 1 SV = 5</td>
<td>4.00</td>
</tr>
<tr>
<td>IFRF2_HUMAN Interferon regulatory factor 2 OS = Homo sapiens GN = IFRF2 PE = 1 SV = 2</td>
<td>4.00</td>
</tr>
<tr>
<td>F205FB_HUMAN Protein FAM208B OS = Homo sapiens GN = FAM208B PE = 1 SV = 1</td>
<td>4.00</td>
</tr>
<tr>
<td>B4D9Y2_HUMAN Sentrin-specific protease 5 OS = Homo sapiens GN = SENPS5 PE = 2 SV = 1</td>
<td>4.00</td>
</tr>
<tr>
<td>KMT2B_HUMAN Histone-lysine N-methyltransferase 2B OS = Homo sapiens GN = KMT2B PE = 1 SV = 1</td>
<td>3.00</td>
</tr>
<tr>
<td>KHYNYN_HUMAN Protein KHYNYN OS = Homo sapiens GN = KHYNYN PE = 2 SV = 3</td>
<td>3.00</td>
</tr>
<tr>
<td>E7EN32_HUMAN Menin OS = Homo sapiens GN = MEN1 PE = 2 SV = 1</td>
<td>3.00</td>
</tr>
<tr>
<td>J3GSB5_HUMAN 60S ribosomal protein L36 OS = Homo sapiens GN = RPL36 PE = 2 SV = 1</td>
<td>3.00</td>
</tr>
<tr>
<td>CEP55_HUMAN Centrosomal protein of 55 kD OS = Homo sapiens GN = CEP55 PE = 1 SV = 3</td>
<td>3.00</td>
</tr>
</tbody>
</table>
tive to WT samples; 13 with significantly decreased mRNA expression and 18 with increased mRNA expression in 
Hcfc2−/− samples (Table 2). These results suggest that HCFC2 facilitates the binding of IRF2 to a variety of target genes to support the functions of IRF2 as both a transcriptional activator and a transcriptional repressor. Because different cell types were used in ChIP-seq and RNA-seq experiments, we note that the overlapping gene set may underestimate the true number of genes coregulated by HCFC2 and IRF2.

Defective host defense caused by Hcfc2 deficiency

Among genes whose expression was affected by HCFC2 and IRF2 deficiencies, a substantial number are known IRGs or contain an IRF-E in their promoter regions. Indeed, IFN-β stimulation induced the transcription of 888 genes in WT BMDMs, among which 153, 132, or 72 genes showed reduced expression in Irf2−/− BMDMs, Hcfc2−/− BMDMs, or in both Irf2−/− and Hcfc2−/− BMDMs. The diminished expression of four IRGs in Hcfc2−/− and Irf2−/− BMDMs was confirmed by quantitative RT-PCR (qRT-PCR; Fig. 7, A–D). Notably, these four genes, Trail (TNF-related apoptosis-inducing ligand), Iigp1 (interferon inducible GTPase 1), Mov10 (Molloney leukemia virus 10), and Ifi47 (IFN-γ-inducible protein 47), as well as other untested IRGs, are known immune response genes involved in defense against parasitic, bacterial, and viral infections, suggesting an important role of Hcfc2 in host defense.

Indeed, when challenged with IAV, 100% of homozygous fls mice died by 12 d after infection; WT mice recovered from IAV infection by day 9 after infection (Fig. 7 E). No significant differences in weight loss were observed between the homozygous fls and WT mice after IAV infection (Fig. 7 F).
**Fls** homozygotes infected with HSV1 developed ataxia and paralysis; all died within 5 d after infection, whereas infected WT mice survived past 8 d after infection (Fig. 7 G). In addition, HSV1-induced IFN-α and IFN-β were reduced in serum from infected homozygous fls mice compared with infected WT mice (Fig. 7, H and I). These data demonstrate that fls mutant mice display susceptibility to multiple virus infections and support that Hcfc2 is required for proper host defense.

**DISCUSSION**

IRF1 and IRF2 regulate Tlr3 transcription by binding to an IRF-E near the transcription start site of Tlr3. We found that HCFC2, a protein with previously unknown function, formed a complex with IRF1 or IRF2 and promoted their binding to the Tlr3 IRF-E and to other gene targets. We hypothesize that the interaction between HCFC2 and either IRF1 or IRF2 may generate a conformational change in the IRF that favors DNA binding. An effect of amino acid substitutions or of protein interactions with the IRF2 C terminus, to which HCFC2 binds, on DNA binding by the N terminus has previously been reported (Childs and Goodbourn, 2003; Prakash and Rath, 2010) and supports this hypothesis. Moreover, a function similar to the one we propose for HCFC2 has been suggested for HCFC1 in its interaction with VP16, an HSV protein that directs the formation of a DNA-binding complex necessary for viral immediate early gene transcription (Kristie and Sharp, 1990). In that system, HCFC1 was required for the formation of a tripartite complex containing HCFC1, VP16, and Oct 1 that associated with viral DNA (Gerster and Roeder, 1988; Katan et al., 1990; Xiao and Capone, 1990). VP16 showed little DNA-binding activity by itself, but this activity was enhanced by a cycle of protein denaturation–partial renaturation or by formation of the VP16–Oct1–HCFC1 complex, both of which are suggestive of conformational change (Marsden et al., 1987; Kristie and Sharp, 1990). HCFC1 bound directly to VP16 in the absence
of DNA, but no direct association between HCFC1 and either DNA or Oct1 was observed (Katan et al., 1990; Kristie and Sharp, 1990; Xiao and Capone, 1990).

By regulating the DNA binding conformation of IRF1 and IRF2, HCFC2 may help these IRFs discriminate between IRF-Es in different genes. Both IRF1 and IRF2 have been shown to distort the conformation of the DNA helix upon binding (Escalante et al., 1998; Fujii et al., 1999), an event that promotes synergistic binding of additional transcriptional regulators; HCFC2 may influence the conformation of IRF1/2-bound DNA. Because IRF2 is an oncogenic protein maintained at low levels in healthy cells (Nguyen et al., 1995), it may rely especially on the support of HCFC2 to stimulate adequate transcription of essential target genes such as Tlr3.

In addition to Tlr3, numerous genes required both HCFC2 and IRF2 for their normal expression. Among them were a large group of IRGs, which aid in the immune response by, for example, directly inhibiting viral components or activating immune cells (Schneider et al., 2014). Although Tlr3−/− mice have been reported to survive as well or better than WT mice infected with influenza or HSV1, respectively (Zhang et al., 2013), Hcfc2fls/fls mice infected with either virus showed significantly reduced survival compared with WT mice. We hypothesize that impaired expression of numerous IRGs, in addition to Tlr3, that require HCFC2 for their IRF2-mediated transcription may account for the increased susceptibility of Hcfc2fls/fls mice to these viruses. Our data suggest that HCFC2 broadly regulates IRF2-dependent transcription and may therefore be necessary for defense against diverse infections.

**MATERIALS AND METHODS**

**Mice**

6-wk- to 6-mo-old male and female mice (Mus musculus) on a pure C57BL/6J background were used in experiments. The Hcfc2fls (designated fls), Hcfc2min (designated min), and Hcfc2sca (designated sca) alleles were generated on a pure C57BL/6J background by ENU mutagenesis (Georgel et al., 2008) and are described at http://mutagenetix.utsouthwestern.edu; they are available from the Mutant Mouse Regional Resource Center. Hcfc2−/− mice were generated by the TALEN-based method on the C57BL/6J background as described previously (Boch et al., 2009; Moscou and Bogdanove, 2009; Sanjana et al., 2012). The Irf2−/− and MyD88−/− mice were gifts from Tak Wah Mak (University of Toronto, Toronto, ON, Canada) and Shizuo Akira (Osaka University, Osaka, Japan), respectively. The Tlr3−/− mice, Msr1−/− mice, and C57BL/10J mice used for positional cloning were purchased from...
The Jackson Laboratory. All targeted knockout mice were backcrossed to the C57BL/6J strain for at least six generations. Controls were WT littermates.

All experimental procedures using mice were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center and were conducted in accordance with institutionally approved protocols and guidelines for animal care and use. Mice were maintained at the University of Texas Southwestern Medical Center, and studies were performed in accordance with institutionally approved protocols. Animals were to be excluded from analysis only if they displayed obvious illness or death (except from HSV1 and influenza infection experiments); these conditions were not observed, and no animals were excluded. No randomization of the allocation of samples or animals to experimental groups was performed.

Positional cloning of \textit{fls}

The index \textit{fls} mutant was outcrossed to C57BL/10J females, and F1 siblings were intercrossed. Bulk segregation analysis was performed as described previously (Xia et al., 2010) using

Table 2. Genes exhibiting reduced association with IRF2 in ChIP-seq and altered transcript levels in RNA-seq for \textit{Hcfc2}−/− samples relative to WT samples

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Genes with lower expression in \textit{Hcfc2}−/− cells</th>
<th>Genes with higher expression in \textit{Hcfc2}−/− cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asah2</td>
<td>Bcl11a</td>
<td>Angpt4</td>
</tr>
<tr>
<td>Enpp2</td>
<td>Fam43a</td>
<td>Cad1</td>
</tr>
<tr>
<td>Gca</td>
<td>H2-Q4</td>
<td>Csf1</td>
</tr>
<tr>
<td>Herpud1</td>
<td>Hfe</td>
<td>Has2</td>
</tr>
<tr>
<td>Ifi44</td>
<td>Ifi44</td>
<td>Has2os</td>
</tr>
<tr>
<td>Rab19</td>
<td>Tlr3</td>
<td>Hmga2</td>
</tr>
<tr>
<td>Tnfrsf14</td>
<td>Zbtb18</td>
<td>Inhba</td>
</tr>
</tbody>
</table>

**Antibodies and reagents**

The following antibodies were used: p38 (catalog no. 9212), phosphorylated (p) p38 (catalog no. 4511), ERK (catalog no. 4695), p-ERK (catalog no. 4370), JNK (catalog no. 9252), p-JNK (catalog no. 9251), Akt (catalog no. 4691), p-Akt (catalog no. 4060), p-STAT1 (catalog no. 7649), TBK1 (catalog no. 3504), p-TBK1 (catalog no. 5483), IxB (catalog no. 4814), Tri-Methyl-Histone H3 (Lys4; catalog no. 9751), and GAP DH (catalog no. 8884; Cell Signaling); mouse TLR3 (catalog no. 14-9032-82; eBioscience); STAT1 (catalog no. sc-346), IRF1 (catalog no. sc-497x), IRF2 (catalog no. sc-498x), and HCFIC2 (catalog no. sc-367211; Santa Cruz); FLAG tag (catalog no. A8592); HA tag (catalog no. M047-7); and Myc tag (catalog no. M215-7; MBL International).

The following reagents were used: Pam3CSK4 and R848 (InvivoGen), poly(I:C) (GE Healthcare), LPS and MALP-2 (ENZO Life Sciences), CpG-ODN 1668 (Sigma-Aldrich), recombinant mouse TNF protein (Life Technologies), mouse TNF ELISA Ready-SET-Go kit (eBioscience), recombinant mouse and human IFN-β (R&D Systems), and mouse IFN-α and IFN-β ELISA kits (PBL Assay Science).

None of the cell lines used (THP1, HEK293T, or NIH-3T3) are listed in the database of commonly misidentified cell lines maintained by the International Cell Line Authentication Committee and National Center for Biotechnology Information Biosample.

**PM response assays**

Mouse PMs were isolated as previously described (Xiao et al., 2009). In brief, mice of the indicated genotypes were injected i.p. with Brewer’s modified thioglycolate (3% wt/vol; BD Biosciences). Macrophages were collected by peritoneal lavage with 5 ml of PBS on day 4 after injection. The PMs were cultured at 37°C in 95% air and 5% CO₂ in DMEM cell culture medium (DMEM containing 10% vol/vol FBS [Gemini Bio Products] and 1% vol/vol penicillin and streptomycin [Life Technologies]).

For TNF and type I IFN production assays, macrophages were preplated onto 96-well plates at a density of 5 × 10⁴ cells/well overnight or for 3 d (specifically for TLR3 signaling). Cells were incubated with varying concentrations of TLR agonists as follows: LPS (125, 250, 500, and 1000 pg/ml), poly(I:C) (12.5, 25, 50, and 100 µg/ml), Pam3CSK4 (12.5, 25, 50, and 100 ng/ml), R848 (2.5, 5, 10, and 20 ng/ml), CpG-ODN 1668 (62.5, 125, 250, and 500 µg/ml), and MALP2 (12.5, 25, 50, and 100 pg/ml), or left unstim-
ulated at 37°C for 4 h. Culture medium was collected for ELISAs. Cells were subjected to an MTT assay (Sigma-Aldrich) for normalization.

For poly(I:C) artificial internalization, macrophages were transfected with DOTAP liposomal transfection reagent (Roche Applied Science) according to the manufacturer’s instructions. 24 h later, culture medium was collected for ELISA.

**qRT-PCR**

Total RNA was extracted and purified with TRizol reagent (Life Technologies) and reverse-transcribed using SuperScript III First-Strand Synthesis SuperMix (Life Technologies) according to the manufacturer’s instructions. Quantitative PCR was performed using the StepOnePlus Real-Time PCR System (Life Technologies). The mRNA levels of the genes of interest were normalized to Gapdh, and relative gene expression was calculated using the 2^{-ΔΔCt} method, where ΔΔCt = ΔCt,sample − ΔCt,reference. The following primers were used for qRT-PCR: 5'-TTGTCTTTCTGCAAGACCTG-3', 5'-CGCAAAGCAGAGGGAGTATTAT-3' (Tlr3); 5'-AGGATTATTGGACTCCACTGA-3', 5'-TAAAGAATGTTTGCTCTGAAGGTTCT-3' (Trail); 5'-TCCTCAAGGACGTGAGTCCGAT-3', 5'-GCCCTGAGGCCGTATTTTAT-3' (Iligp1); 5'-AGACCGCTGCGCAGAGTCAGAAGTTCT-3', 5'-GAAGGCTGAATGAACTCGTACCA-3' (Ifi47); 5'-TGGAATGACCTGGACCTGAGACAGA-3', 5'-GCTCAGTCTTCAATCATTCCATA-3' (Hcfc2); and 5'-TGCTGAGTACAGTTACCAGG-3', 5'-GGCCCTCCTAGGCTACAT-3' (Gapdh).

**Immunoprecipitation**

293T cells were transfected with the designated expression vectors using PolyJet in vitro DNA transfection reagent (Sigma-Aldrich) for normalization.

---

Figure 7. **HcFC2 is required for host defense.** (A–D) qRT-PCR measurement of Trail (A), Iigp1 (B), Mov10 (C), and Ifi47 (D) normalized to Gapdh in untreated or IFN-β-stimulated BMDMs from Irf2−/−, Hcfc2−/−, and WT (+/+ ) mice. (E–I) Homozygous fls mice and their WT littermates were challenged with 10⁵ pfu IAV per mouse (E and F) or 10⁷ pfu HSV1 per mouse (G–I). Kaplan–Meier survival curve (E) and body weight as a percentage of initial body weight after IAV infection (F). Kaplan–Meier survival curve (G) and IFN-α (H) or IFN-β (I) in the serum 4 h after HSV1 infection. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001, unpaired Student’s t test (A–D, H, and I), log-rank (Mantel-Cox) test (E and G). Data represent mean ± SEM; n = 3 mice (A–D) and n = 5 mice per genotype (E–I). Results are representative of three independent experiments.
naGen). 48 h later, cell lysates were prepared in IPH lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, and 0.5% NP-40). The lysates were then incubated with magnetic bead–conjugated Myc antibody (MBL International) on a rotator at 4°C overnight. After three washes in cold lysis buffer, proteins bound to the beads were dissociated by boiling in Laemmli SDS sample buffer (Alfa Aesar), separated by SDS-PAGE, and transferred to a membrane. Immunoblottting was performed using the indicated antibodies.

ChIP
ChIP assays were performed as previously reported (Koike et al., 2012; Takahashi et al., 2015). In brief, to prepare chromatin, MEFs were dual cross-linked using 2 nM EGS (20 min) in PBS followed by formaldehyde (1% vol/vol final concentration, 8 min). Dual–cross-linked nuclei were resuspended in 1 ml of lysis buffer (20 mM Tris, pH 8, 150 mM NaCl, 2 mM EDTA, pH 8, 0.1% wt/vol SDS, 1% vol/vol Triton X-100, 1 mM PMSF; and Roche complete EDTA free protease inhibitor cocktail; Whyte et al., 2013) and sonicated nine times for 30 s at 4°C using a Covaris E220 ultrasonicator (150 V peak power, duty factor 10, 200 cycles/burst). 15 µg soluble chromatin was incubated with 1 µg IRF2 antibodies in IP buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% vol/vol Triton X-100, 0.1% wt/vol sodium deoxycholate, 1 mM PMSF, and PI cocktail) overnight at 4°C. 50 µl Dynabeads Protein G was then added and incubated for 2 h at 4°C. After extensive washes, communoprecipitated DNA fragments were eluted, reverse cross-linked, and then purified with the Qiaquick PCR purification kit (QIAGEN). For quantification of coprecipitated DNA, samples were subjected to amplification by using the following primers: 5′-GGG CCT CCT GGG ATT -3′, 5′-GGC TCT GAA GCC AGA -3′. The sequences of the oligonucleotide probes were as follows: mouse Tlr3 IRF-E sense oligo, biotin-5′-TCA GCC TGA AAG TGA AAC TTA AGT TGAG-3′; human TLR3 IRF-E sense oligo, biotin-5′-ACG TTT ACT -3′; 5′-GCCCTACCCCATTTGATTT-3′ (Gapdh). Amplification of Gapdh from the unprecipitated chromatin was used to control the amount of input material.

For ChIP-seq, library preparation for next-generation sequencing was based on the Illumina TruSeq ChIP Sample Preparation Kit. In brief, ChIP DNA (40 µl) was end-repaired, and ~150-bp fragments were size-selected using AmpureXP beads followed by adenylation and adapter ligation. After adapter ligation, samples were amplified by PCR and purified with AmpureXP beads and validated on the Agilent 2100 Bioanalyzer. Finally, samples were quantified by Qubit, normalized, and pooled to run on the Illumina NextSeq 500 (150 cycles).

MEF ChIP-seq sample reads from the Illumina NextSeq 500 were mapped to the mm10 genome assembly from the University of California, Santa Cruz, using Bowtie2 v.2.1.0 (Langmead and Salzberg, 2012). Mapped reads containing mapping quality scores >10 were filtered using samtools. Duplicate reads were removed followed by extending the remaining reads to fragment size. Coverage tracks were made by normalizing to 10 million reads. ChIP peaks for MEF samples were identified from uniquely mapped reads without duplicates using MACS (Zhang et al., 2008b) with parameters ‘–p 1e-5 --size min --nomodel True --bspace=10’ and relevant input chromatin samples as control data. MACS peaks were then subdivided using PeakSplitter with a valley cutoff of 0.7 and were later filtered for summit height >6. To construct a master peak list from the four MEF samples (two Hcfc2−/− and two WT), the peaks obtained after PeakSplitter and summit height filtering were merged and compared for overlaps, and the peak with highest summit value was chosen if the summit coordinates were within the fragment size estimate of 192 bp. Raw reads in the master peak regions were quantified using HOMER’s (Heinz et al., 2010) annotatePeaks.pl program. The resultant counts matrix was used as input with DESeq2 (Love et al., 2014) and master peak regions with fold change ≥2 and false discovery rate <0.05 were considered differentially enriched and significant. For motif analysis of the 381 IRF2-binding sites (Table S3), binding peak summits ±100 bp were used as input to identify 12-bp motifs using HOMER. HOMER selected a random background sequence with similar GC content to test for statistical significance.

IRF1 and IRF2 DNA-binding activity by gel shift assay and DNA affinity chromatography pulldown
Recombinant FLAG tagged IRF1, IRF2, and HCFC2 were purified with magnetic bead–conjugated FLAG antibody (Sigma-Aldrich) after overexpression in 293T cells. Cell nuclear lysates were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific). Gel shift assays were performed using the LightShift Chemiluminescent EMSA kit (Pierce), according to the manufacturer’s instructions. In brief, 1 µg IRF1, 1 µg IRF2, and 0.5 µg HCFC2 recombinant protein or 3 µg nuclear lysate were used in binding reactions, which were further analyzed by electrophoresis through a native 6% polyacrylamide gel. Competitive assays were also performed by addition of 50-fold excess of unlabeled probe at room temperature for 5 min before the addition of the labeled probe. Supershift assays were performed by addition of 2 µg antibodies at room temperature for 15 min after binding reaction. The sequences of the oligonucleotide probes were as follows: mouse Tlr3 IRF-E sense oligo, biotin-5′-TCA GCC TGA AAG TGA AAC TTA AGT TGAG-3′; human TLR3 IRF-E sense oligo, biotin-5′-ACG TTT ACT -3′; 5′-GCCCTACCCCATTTGATTT-3′ (Gapdh). Amplification of Gapdh from the unprecipitated chromatin was used to control the amount of input material.
England Biolabs) were added to the reaction and incubated at 4°C for 1 h. The protein–DNA–streptavidin complex was washed three times with binding buffer and loaded onto an SDS gel. Detection of IRF1, IRF2, and HCF2C proteins was performed by immunoblotting.

**Virus challenge**

Age-matched mice (usually 8–12 wk old) were infected with either HSV1 (strain KOS, provided and prepared by the laboratory of Z.J. Chen, University of Texas Southwestern Medical Center, Dallas, TX) at 10⁷ pfu per mouse via retro-orbital injection or IAV (strain A/H1N1/CA/2009, prepared and provided by the laboratory of J.-L. Casanova, The Rockefeller University, New York, NY) at 10⁵ pfu per mouse via intranasal inoculation. In the HSV1 challenge, the viability of the infected mice was monitored daily for 14 d. 4 h after HSV1 infection, serum was collected and subjected to IFN-β and IFN-α ELISA assays per the manufacturer’s instructions. In β herpesvirus infection, serum was collected and subjected to IFN-α and infected mice was monitored daily for 14 d. 4 h after HSV1 infection, serum was collected and subjected to IFN-β and IFN-α ELISA assays per the manufacturer’s instructions. In the IAV challenge, the viability and weight loss were monitored daily for 21 d after inoculation.

**High-throughput RNA-seq and bioinformatics analysis**

BMDMs were obtained through a macrophage colony-stimulating factor–induced differentiation protocol as previously described (Zhang et al., 2008a). Total RNA was prepared using the RNeasy Plus Mini kit (QIAGEN) according to the manufacturer’s instructions. Paired-end 2 × 100 bp sequencing was performed using an Illumina HiSeq 2500. Reads were demultiplexed and converted to fastq format using CASAVA v.1.8.2. Reads were mapped using TopHat2 (http://ccb.jhu.edu/software/tophat/index.shtml; Kim et al., 2013), and differential expression was examined with Cuffdiff, a part of the Cufflinks package (http://cole-trapnell-lab.github.io/cufflinks/; Trapnell et al., 2013). Gene Ontology annotations of differentially expressed genes (P < 0.05), between WT and either Hcfc2−/− or Irf2−/− mice, were annotated using DAVID (the Database for Annotation, Visualization and Integrated Discovery) bioinformatics resources v.6.7 (https://david.ncifcrf.gov; Huang et al., 2007). Heat maps and visualizations were created using the R packages CummeRbund (http://compbio.mit.edu/cummeRbund/), ggplot2 (http://ggplot2.org), and heatmap.2 from gplots (https://mran.microsoft.com/package/gplots/).

**Statistical analysis**

An unpaired Student’s t test was used for comparisons between two unpaired experimental groups. For comparisons of differences in responses affected by two factors, two-way ANOVA was used. The log-rank test (Mantel–Cox) was used for comparisons of survival curves. Data represent mean ± SEM in all graphs depicting error bars. The statistical significance of differences between experimental groups was determined using GraphPad Prism v.6. P < 0.05 was considered statistically significant. P-values are indicated as follows: *P < 0.05; **P < 0.01; ***P ≤ 0.001; and ****P ≤ 0.0001; ns, not significant with P > 0.05. No prespecified effect size was assumed, and in general three to five animals or replicates for each genotype or condition were used in experiments; this sample size was sufficient to demonstrate statistically significant differences in comparisons. The investigator was not blinded to genotypes or group allocations during any experiment.

The phenotypic performance of mice (C57BL/6J) and primary cells of these mice is expected to follow a normal distribution, as has been observed in large data sets from numerous phenotypic screens conducted by our group. Variation within each data set obtained by measurements from mice or primary cells was assumed to be similar between genotypes, because all strains were generated and maintained on the same pure inbred background (C57BL/6J); experimental assessment of variance was not performed.

**Online supplemental material**

Fig. S1 contains the full-size gel and immunoblot images from all figures. Fig. S2 shows the mapping of the interacting domains of IRF2 and HCF2C. Table S1 shows the sequence statistics for ChIP-seq experiments. Table S2 is the master peak list for IRF2 ChIP-seq experiments. Table S3 is the list of 381 differentially enriched master peaks for IRF2 ChIP-seq experiments. Tables S1–S3 are included as Excel files.

**ACKNOWLEDGMENTS**

The authors thank Peter Jurek for expert assistance with figure preparation. This work was supported by National Institutes of Health grant U19 AI100627. The authors declare no competing financial interests.


Submitted: 27 September 2016
Revised: 13 July 2017
Accepted: 16 August 2017

**REFERENCES**


