IMMUNOLOGY

GPER1 is required to protect fetal health from maternal inflammation

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Type I interferon (IFN) signaling in fetal tissues causes developmental abnormalities and fetal demise. Although pathogens that infect fetal tissues can induce birth defects through the local production of type I IFN, it remains unknown why systemic IFN generated during maternal infections only rarely causes fetal developmental defects. Here, we report that activation of the guanine nucleotide–binding protein–coupled estrogen receptor 1 (GPER1) during pregnancy is both necessary and sufficient to suppress IFN signaling and does so disproportionately in reproductive and fetal tissues. Inactivation of GPER1 in mice halted fetal development and promoted fetal demise, but only in the context of maternal inflammation. Thus, GPER1 is a central regulator of IFN signaling during pregnancy that allows dynamic antiviral responses in maternal tissues while also preserving fetal health.

When pathogenic infections occur during pregnancy, the maternal immune system must respond and suppress pathogen replication without targeting the genetically heterologous fetus (*1*, *2*). In some cases, however, including during in utero infections, consequential fetal inflammation cannot be avoided, leading to birth defects and fetal demise (*3*–*5*). In particular, fetal type I interferon (IFN) signaling can be a major driver of developmental abnormalities (*4*, *5*). Many infections that are common during pregnancy, such as influenza A virus (IAV), induce systemic type I IFN and could cause fetal IFN signaling without local viral replication (*6*, *7*). Yet maternal IAV infections are rarely linked to birth defects (*8*, *9*). Thus, a mechanism may exist that allows IFN signaling in infected tissues while preventing maternal type I IFN from initiating signaling in the fetus.

To identify IFN regulators that could mediate differential IFN control across tissues, we performed genome-wide CRISPR-Cas9 screens in a human epithelial cell line with an IFN response reporter (fig. S1). After IFN treatment of the reporter cells and removal of the cytokine, we collected cells that aberrantly maintained fluorescence to identify the proteins required to down-regulate IFN signaling (Fig. 1A). Through bioinformatic analysis, we identified a number of genes that were enriched above the nontargeting sgRNA controls, including, as expected, proteasomal subunits that directly prevent green fluorescent protein degradation (Fig. 1B and table S1).

We used screen-enriched single-guide RNAs (sgRNAs) to target nine genes selected from among our top hits and saw that the targeting of six genes significantly prolonged IFN signaling (table S2). One of the validated screen hits, guanine nucleotide–binding protein–coupled estrogen receptor 1 (*GPER1*, also known as *GPR30*), is a nonclassical estrogen receptor (*10*) that was likely activated by fetal calf serum–derived estrogen during our screen. Because estrogen concentrations increase greatly during pregnancy (*11*, *12*), GPER1 had the potential to link pregnancy hormone levels to regulation of the IFN response. Because our initial *GPER1* validation was performed with a polyclonal population, we next verified our IFN reporter results with a clonal line (Fig. 1, C and D). We then made use of a GPER1-specific inhibitor, G15, which competitively blocks estrogen binding (*13*). In a dose-dependent manner, treatment with the inhibitor prevented appropriate down-regulation of the IFN response reporter (Fig. 1, E and F) as well as endogenous IFN-stimulated gene (ISG) mRNA transcripts (Fig. 1G). As expected, in our clonal GPER1 sgRNA line, G15 treatment did not significantly alter IFN signaling (Fig. 1H).

To determine whether GPER1 activity is sufficient to suppress IFN signaling, we overexpressed GPER1 (Fig. 2A). Without major alterations to cell viability (Fig. 2B and fig. S2), the IFN response was suppressed as measured by the IFN reporter as well as endogenous ISG RNA and protein levels (Fig. 2, C to H). We also used the GPER1 agonist G1, which specifically activates GPER1 (*14*). At a concentration that did not induce apparent cellular toxicity, G1 significantly reduced IFN signaling across a range of IFN treatment concentrations (Fig. 2, I and J). Finally, we tested individual estrogens to elucidate which natural GPER1 ligands can suppress IFN signaling. Consistent with previous reports describing GPER1 activation (*15*), estradiol (E2) and, to a much smaller extent, estrone (E1) could suppress the IFN response reporter, whereas estriol (E3) could not (Fig. 2K).

To determine which tissues might have biologically relevant GPER1 activity, we performed GPER1 protein immunoblots on tissues from pregnant mice. Consistent with the reports of GPER1 distribution in humans (*16*), expression was highest in the reproductive and fetal tissues, including the placenta (Fig. 3A). Because type I IFN signaling in the placenta has been implicated as a key determinant of fetal fate, we performed immunohistochemical analysis for the localization of GPER1 in the placenta. Although GPER1 was expressed in all of the major placental zones, we observed high expression in the cells that line the vascular labyrinth (Fig. 3B). Despite differences in blood supply architecture, GPER1 was also highly expressed in the cells that contact the maternal blood supply in human placental samples (fig. S3).

Elevated estrogen concentrations during pregnancy, combined with differential GPER1 expression across the maternal periphery and fetal tissues, suggested that GPER1 may mediate tissue-specific regulation of IFN signaling during pregnancy. We therefore used a murine pregnancy model of IAV infection, administering either the GPER1 inhibitor G15 or a vehicle control (Fig. 3C). Presumably because of relatively lower GPER1 expression in the respiratory tract, drug treatment had no major effects on maternal morbidity, the infectious viral titer, the lung transcriptional response, or the pulmonary inflammatory cytokine signature (Fig. 3, D to F, and figs. S4 and S5). Influenza disease in nonpregnant mice, at least in the C57BL/6 background, was similarly unaffected by G15 treatment (fig. S6).

By contrast, the placental response to maternal IAV infection (fig. S7) was significantly altered by G15 inhibition of GPER1 (Fig. 3G). These transcriptional changes were not attributable to G15 treatment allowing for viral dissemination from the respiratory tract (Fig. 3H). Instead, the inhibition of GPER1 during maternal infection led to the up-regulation of many, but not all, canonical ISGs and IFN-induced cytokines (*17*) such as CCL3 and CCL5, but not *Ifnb* itself (Fig. 3I and fig. S8). Our inability to detect the up-regulation of all ISGs was likely due to constitutive type III IFN responses in some regions of the placenta (*18*). Additionally, IFN treatment of primary human placental explants with and without G15 also led to increased ISG expression (fig. S9). Many non-IFN–regulated genes were also affected during G15 treatment and infection. Gene ontology of the suppressed genes revealed the enrichment of pathways involved in blood vessel development and angiogenesis (figs. S10 and S11), consistent with the reports of type I IFN signaling suppressing angiogenesis (*19*). As expected from the transcriptional data, we also observed an obvious attenuation or degradation of the placental endothelium when G15 was

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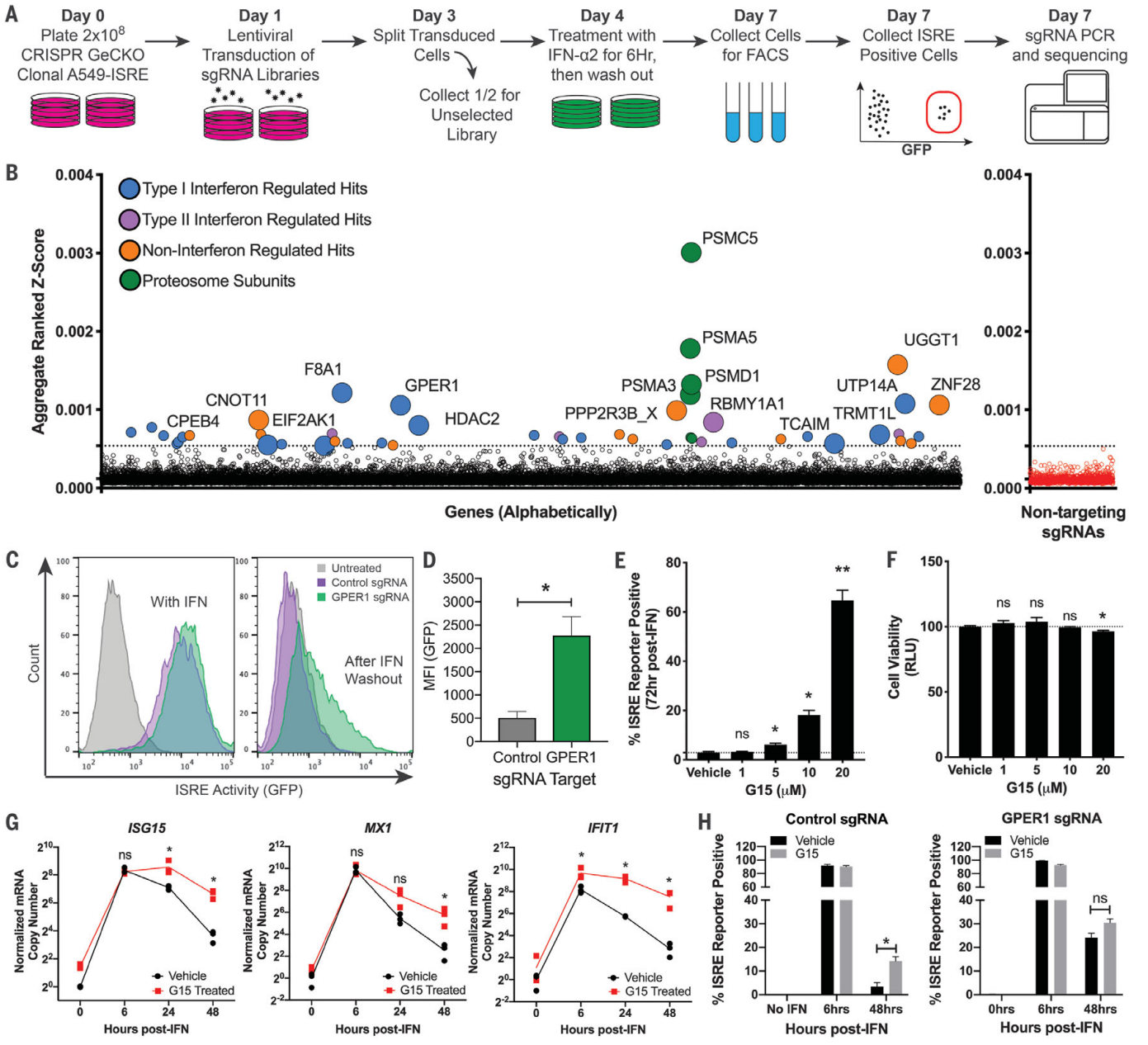

Fig. 1. A CRISPR screen identifies GPER1 as a negative regulator of the type I IFN response. (**A**) Schematic of the CRISPR genome-wide knockout screens. FACS, fluorescence-activated cell sorting; PCR, polymerase chain reaction. (**B**) Graphical representation of the screening results with candidate regulators (left) and the nontargeting controls (right). (**C**) Representative flow cytometry histograms of the IFN response during IFN-α2 treatment (left) and 48 hours after IFN-α2 removal (right) in the indicated cell lines. (**D**) Quantification of the mean fluorescence intensity from (C) at 48 hours after IFN-α2 treatment, sample size $n = 3$. GFP, green fluorescent protein; MFI, mean fluorescence intensity. (**E**) Percentage of IFN-stimulated response element (ISRE)–reporter–positive cells 72 hours after IFN-α2 treatment with the indicated concentrations of the GPER1 antagonist G15, $n = 3$. (**F**) Normalized cellular viability of the treatments in (E), $n = 3$. RLU, relative luciferase units. (**G**) Quantitative reverse transcription PCR (qRT-PCR) analysis of the indicated ISGs in vehicle- or G15-treated A549 cells after treatment with IFN-α2, $n = 3$. (**H**) Percent of the indicated cell lines that were ISRE reporter–positive at the indicated times after IFN-α2 treatment with or without G15 treatment, $n = 3$. All data are representative of at least two independent experiments. For all panels, error bars indicate the SEM, and statistical analyses were performed by means of unpaired Student's *t* tests. $^*P \le 0.05$; $^{**}P \le 0.001$; ns, not significant.

administered during IAV infection (Fig. 3, J and K, and fig. S12).

Next, we asked whether neonatal viability would be affected by these placental changes (Fig. 4A). Although again, there was no observable difference in maternal disease (Fig. 4, B and C), pup birth weight was modestly reduced by either viral infection or G15 treatment alone (Fig. 4D), which is consistent with a role for GPER1 in normal uterine function (*20*) and the effects of maternal influenza on fetal birth weight (*12*). However, the most severe reduction in birth weight was observed when G15 treatment occurred in the presence of IAV infection (Fig. 4D). Furthermore,

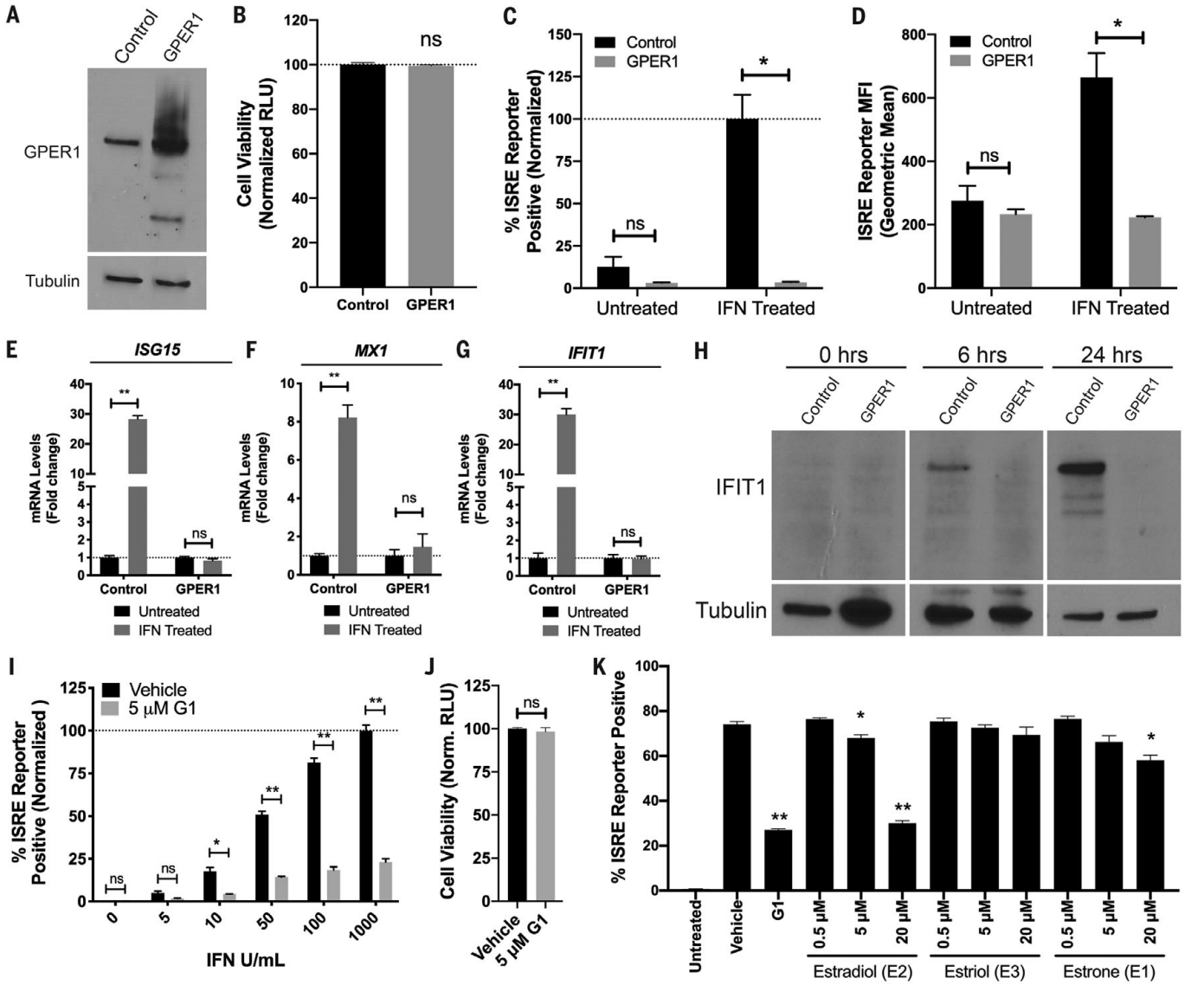

Fig. 2. GPER1 activity is sufficient to suppress the type I IFN response. (**A**) Protein immunoblots of GPER1 and tubulin in control or GPER1-overexpressing 293T cells. (**B**) Viability of 293T cells 18 hours after transfection with either a control or GPER1 expression plasmid, $n = 3$. (**C**) Percentage of 293T cells positive for the ISRE reporter after expression of the indicated protein, $n = 3$. (**D**) Quantification of the mean fluorescence intensity from (C). (**E** to **G**) qRT-PCR analysis of the indicated ISGs after IFN-α2 treatment of 293T cells expressing GPER1 or a control protein, $n = 3$. (**H**) Protein immunoblots of IFIT1 and tubulin after IFN-α2 treatment in 293T cells. (**I**) Percentage of reporter-positive ISRE-A549 cells after IFN-α2 and vehicle or G1 treatment, $n = 3$. (**J**) Cellular viability from (I), $n = 3$. (**K**) Percent of reporter-positive ISRE-A549 cells after treatment with IFN-α2 and the indicated concentrations of estradiol, estriol, or estrone, $n = 3$. All data are representative of at least two independent experiments. For all panels, error bars indicate SEM, and statistical analyses were performed by means of unpaired Student's t tests. $^{*}P \leq 0.05$; $^{**}P \leq 0.001$; ns, not significant.

although the number of pups in each litter was similar across treatment groups, only those pups born to IAV-infected, G15-treated dams were predominantly stillborn (Fig. 4, E to G). Analogous experiments using *Gper1*$^{-/-}$ mice revealed similar viability phenotypes (fig. S13).

Making use of immunocompetent Zika virus (*21*) and influenza B virus mouse pregnancy models, we again observed that G15 treatment exacerbated virus-induced fetal developmental defects (figs. S14 and S15). As a nonviral approach to induce systemic inflammation, we also treated pregnant dams with the viral RNA mimic polyinosinic:polycytidylic acid poly(I:C). Although this treatment by itself negatively affected fetal development, we could exacerbate the effects with G15 and, conversely, promote neonatal viability with the GPER1 agonist G1 (fig. S16). Finally, to experimentally link GPER1 activity to control of IFN signaling, we treated pregnant mice with poly(I:C) in the presence of either control or IFN alpha and beta receptor (IFNAR)–blocking antibodies (Fig. 4H). Administration of the IFNAR-blocking antibody mostly suppressed the negative effects of poly(I:C) and G15 on fetal development (Fig. 4, I and J). Thus, GPER1 activity is largely dispensable even when maternal inflammation is present, provided that type I IFN signaling has been blocked.

We propose a model whereby GPER1 is a central regulator of type I IFN signaling during pregnancy that protects fetal health without compromising peripheral maternal IFN responses. The effects of systemic or "nonlocal" type I IFN have not generally been

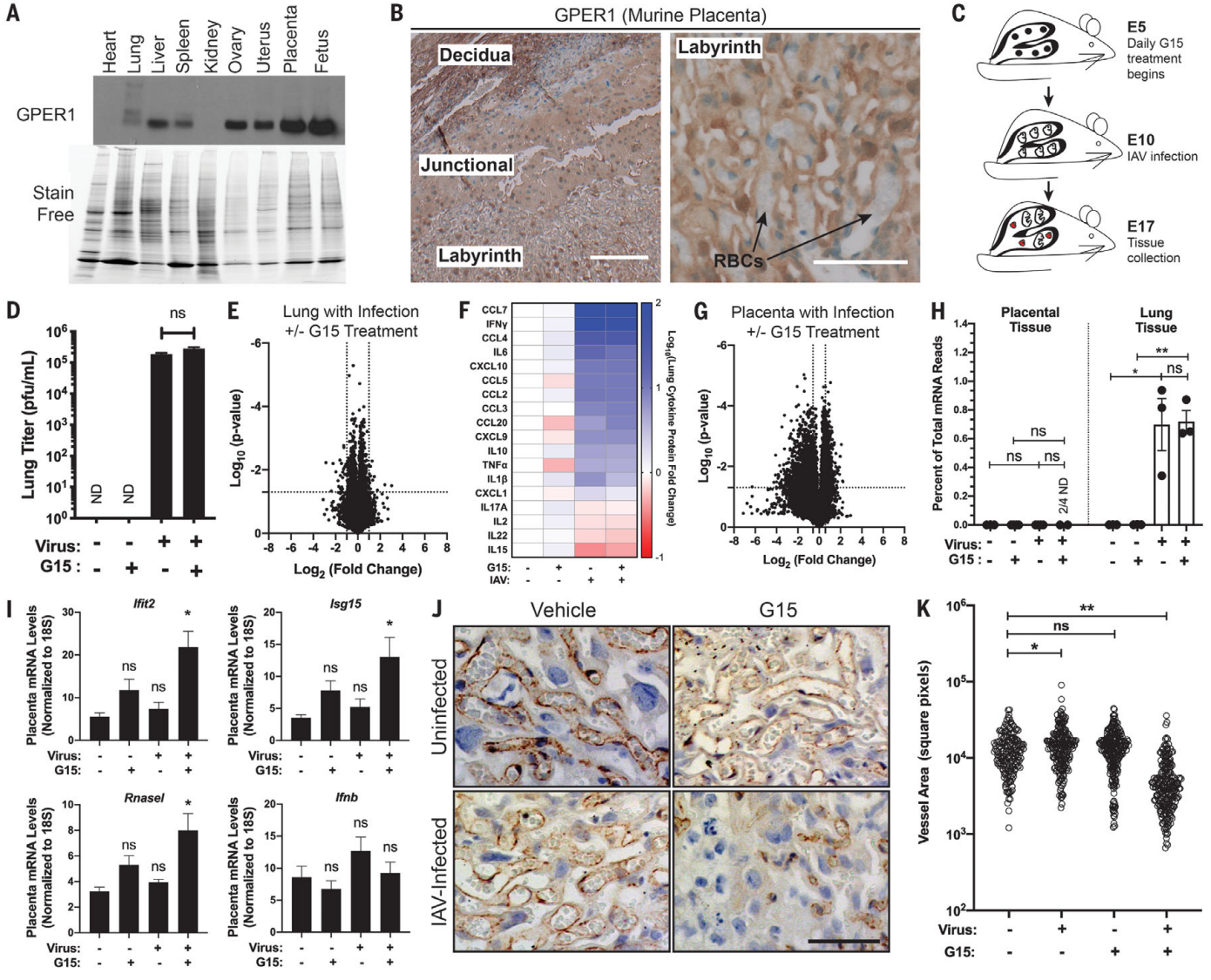

Fig. 3. GPER1 mediates placenta tissue–specific IFN regulation during an influenza virus infection. (**A**) Protein immunoblot for GPER1 on the indicated samples collected from one animal, representative of two mice. (**B**) Representative (n = 4) immunohistochemical staining for GPER1 expression in the mouse placenta. Scale bars, 200 μm (left) and 75 μm (right). RBC, red blood cell. (**C**) Experimental diagram related to panels (D) to (K). E5, embryonic day 5. (**D**) Lung IAV titers from pregnant dams, treated as indicated, 7 days after infection, n = 3. (**E**) Volcano plot of RNA sequencing from the lungs of infected pregnant dams ± G15 treatment. (**F**) Heatmap comparing the relative protein levels of cytokines in lungs described in (D). (**G**) Volcano plot of RNA sequencing from the placentas of infected pregnant dams ± G15 treatment. (**H**) Percent of total RNA sequencing reads that align to the A/Puerto Rico/8/1934 genome from the indicated tissues and treatments, $n \geq 3$ samples analyzed. (**I**) qRT-PCR analysis of ISG mRNA from the placental samples used for RNA sequencing, $n \geq 3$. (**J**) Representative images of CD31 staining of the placental labyrinth from the indicated treatments. Scale bar, 50 μm. (**K**) Quantification of the blood vessel area from the CD31 staining described in (J), $n \geq 180$. For panels (A) and (B), data are representative of at least two independent experiments. In panels (D) to (F) and (H), samples were collected from $n \geq 3$ independently treated animals per group and analyzed or sequenced once. In panels (G to K), $n \geq 3$ placentas were collected from multiple pregnancies and analyzed or sequenced once. For all panels, error bars indicate SEM, and statistical analyses were performed by means of unpaired Student's t tests except for panel (K), which used one-way analysis of variance (ANOVA) and post hoc Dunnett's multiple comparisons test. $^*P \leq 0.05$; $^{**}P \leq 0.001$; ND, not detected; ns, not significant.

appreciated as an important mediator of fetal disease. Our data argue that without GPER1-mediated suppression of IFN signaling, many maternal infections would have notable consequences for fetal health. Recent studies have highlighted the deleterious consequences for placental vasculature and syncytiotrophoblast formation after fetal type I IFN signaling (*4*, *5*). Our findings expand upon those results by identifying a regulatory mechanism for fetal IFN signaling.

Nevertheless, a number of important questions remain unresolved. First, the mechanism by which GPER1 prevents IFN signaling remains unknown. GPER1 activation has been reported to alter cyclic adenosine monophosphate levels, mediate extracellular signal–regulated kinase (ERK) 1/2 activation through epidermal growth factor receptor (EGFR) signaling, and activate the phosphatidylinositol 3-kinase (PI3K)–Akt pathways (*22*). At least in our in vitro system, treatment with the well-characterized EGFR and PI3K inhibitors erlotinib and LY294002 failed to reverse the G1-mediated

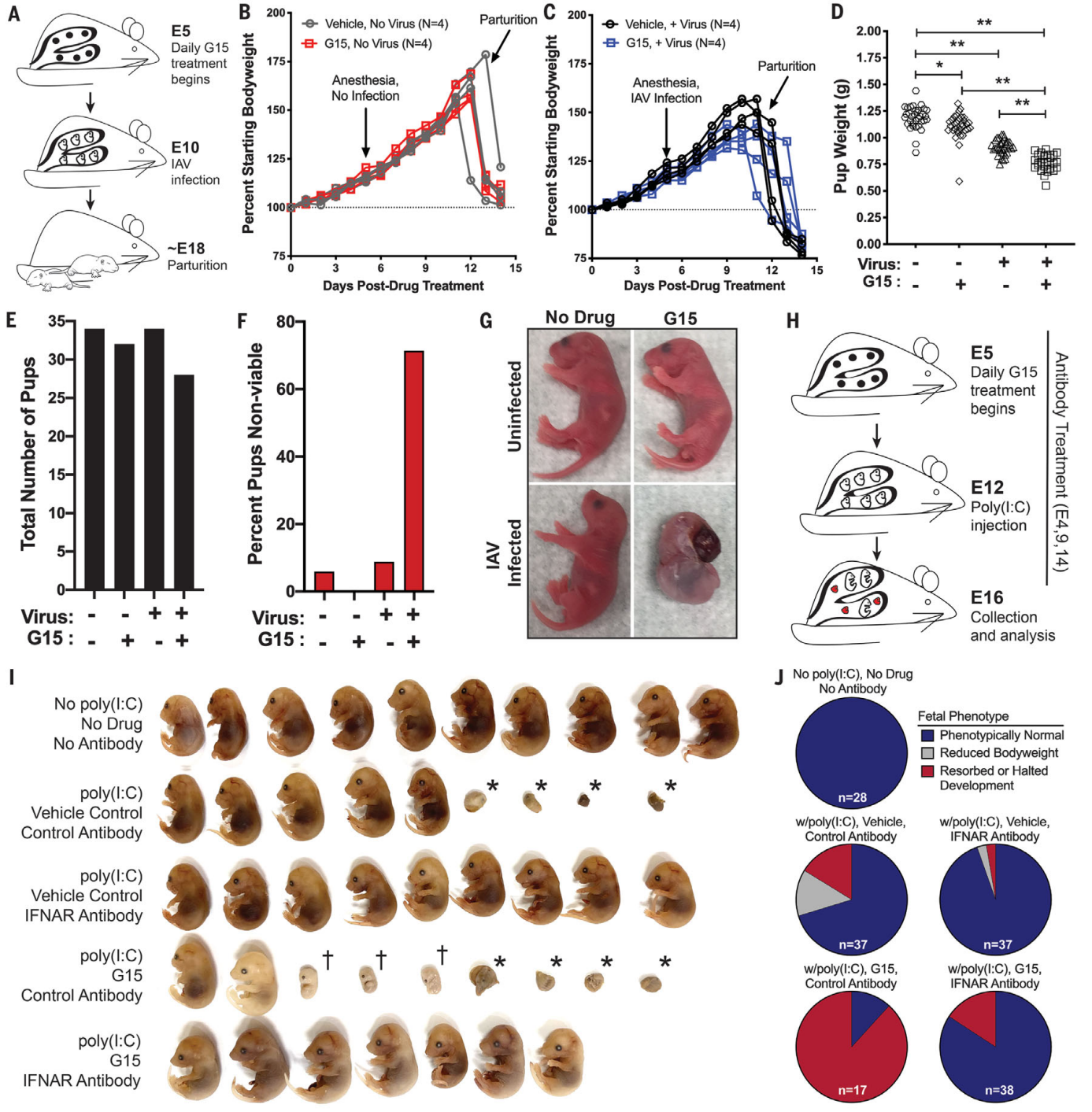

Fig. 4. GPER1 protects fetal development through its regulation of the type I IFN signaling pathway. (**A**) Experimental diagram related to panels (B) to (G). (**B** and **C**) Bodyweight changes of vehicle- or G15-treated, uninfected (B) or IAV-infected (C) pregnant dams. (**D**) Weight in grams of pups immediately after birth from the indicated treatment groups, $n \geq 25$, $^{*}P \leq 0.05$, $^{**}P \leq 0.001$ as per one-way ANOVA and post hoc Tukey's multiple comparisons test. (**E**) Total number of pups from (B) and (C). (**F**) Percentage of pups scored nonviable from (E). (**G**) Images of representative pups from each of the indicated treatment groups. (**H**) Experimental diagram related to panels (I) to (J). (**I**) One representative litter from each of the indicated treatment groups collected on E16. Resorbed fetuses are marked with * and developmentally halted fetuses with †. (**J**) Pie charts indicating the total number of fetuses analyzed and the developmental phenotypes, related to panel (I). All results are representative of at least two independent experiments.

suppression of IFN signaling, potentially implicating other pathways downstream of GPER1 (fig. S17). Also, alterations in GPER1 activity may induce changes in classical estrogen receptor signaling, which could then ultimately influence IFN regulation. Additionally, it will be important to understand why the GPER1-mediated suppression of IFN signaling is insufficient to protect from damage during infection with pathogens that replicate in fetal tissues (*23*). Future studies will be required to definitively answer this question, but we

hypothesize that GPER1 activity can be overwhelmed by relatively high concentrations of IFN at the sites of active pathogen replication.

Finally, on the basis of our experiments with G1, one might speculate that the therapeutic activation of GPER1 signaling could be used to protect fetal health. Although activation of the classical estrogen receptors can influence adult influenza disease (*24*), GPER1 can be targeted independently of the other estrogen receptors (*13*, *14*). Thus, without compromising maternal immunity, the induction of GPER1 activity may be able to prevent a range of developmental conditions linked to maternal infection and/or fetal inflammation (*25*), independent of their underlying etiologies, by targeting the commonality of inappropriate type I IFN signaling.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
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MDAR Reproducibility Checklist
Data S1

View/request a protocol for this paper from *Bio-protocol*.

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GPER1 in utero to the rescue!

Several common pathogens, including influenza A virus (IAV), can activate systemic type I interferon (IFN) signaling during pregnancy. Such infections would be expected to cause birth defects and fetal mortality, but maternal IAV infections rarely produce such effects, suggesting the presence of a protective mechanism in fetal tissues. Harding *et al.* used a CRISPR screen to uncover IFN regulators that can mediate differential IFN control across tissues in human cell lines. They found that G protein–coupled estrogen receptor 1 (GPER1), which is expressed in fetal tissues, acts as a protective suppressor of IFN responses in the placenta during maternal infection. In a mouse model, pharmacological activation of GPER1 shielded fetuses from maternal inflammation. Activation of GPER1 might be promising therapeutically to protect the fetus from both maternal and fetal infections.

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