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Apoptotic caspases suppress an MDA5-driven IFN response during productive replication of human papillomavirus type 31

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Human papillomaviruses (HPVs) infect the basal proliferating cells of the stratified epithelium, but the productive phase of the life cycle (consisting of viral genome amplification, late gene expression, and virion assembly) is restricted to the highly differentiated suprabasal cells. While much is known regarding the mechanisms that HPVs use to block activation of an innate immune response in undifferentiated cells, little is known concerning how HPV prevents an interferon (IFN) response upon differentiation. Here, we demonstrate that high-risk HPVs hijack a natural function of apoptotic caspases to suppress an IFN response in differentiating epithelial cells. We show that caspase inhibition results in the secretion of type I and type III IFNs that can act in a paracrine manner to induce expression of interferon-stimulated genes (ISGs) and block productive replication of HPV31. Importantly, we demonstrate that the expression of IFNs is triggered by the melanoma differentiation-associated gene 5 (MDA5)–mitochondrial antiviral-signaling protein (MAVS)–TBK1 (TANK-binding kinase 1) pathway, signifying a response to double-stranded RNA (dsRNA). Additionally, we identify a role for MDA5 and MAVS in restricting productive viral replication during the normal HPV life cycle. This study identifies a mechanism by which HPV reprograms the cellular environment of differentiating cells through caspase activation, co-opting a non-death function of proteins normally involved in apoptosis to block antiviral signaling and promote viral replication.

HPV | life cycle | IFN | caspase | cancer

Significance

Persistent infection with high-risk human papillomaviruses (HPVs) is associated with the development of multiple human cancers. Evasion of an antiviral innate immune response is key to viral persistence and completion of the life cycle. In this study, we demonstrate that apoptotic caspase activity is pivotal in suppressing an interferon (IFN) response driven by the melanoma differentiation-associated gene 5 (MDA5)–mitochondrial antiviral-signaling protein (MAVS) RNA sensing pathway during productive replication of high-risk HPV31. These studies reveal insight into how HPV controls innate immunity upon differentiation and identify a role for RNA in activating the cell-intrinsic immune response during productive replication.

Human papillomaviruses (HPVs) are small DNA viruses that exhibit a strict tropism for epithelial cells. Persistent infection with HPVs termed high risk (e.g., HPV16, HPV18, HPV31, HPV45) is a major risk factor for the development of multiple human malignancies, most notably cervical cancer (1, 2). However, high-risk HPVs are also associated with an increasing number of head and neck cancers (3). The ability of HPV to escape from immune surveillance is key to viral persistence. One mechanism by which HPV achieves this is by linking its life cycle to the differentiation status of the host cell keratinocyte (4). Upon infection of the basal cells of the stratified epithelium, HPV undergoes a transient amplification process, resulting in 50 to 100 episomes per cell that are stably maintained in basal, undifferentiated cells by replicating once per cell cycle along with cellular DNA (5). Viral replication is accompanied by low-level expression of the early genes (e.g., E1, E2, E6, E7). The productive phase of the HPV life cycle is triggered by epithelial differentiation, resulting in amplification of viral genomes to hundreds to thousands of copies per cell and activation of late gene expression, including E1^E4 and the highly immunogenic capsid genes L1 and L2, which facilitate virion assembly (1). While the induction of late gene expression is not strictly dependent on productive viral replication, the amplification of viral genomes serves to enhance the accumulation of late viral transcripts (6). The uppermost layers of the stratified epithelium are less prone to immune surveillance than the basal layer, and the restriction of high levels of viral gene expression, viral DNA, and virion assembly/release to differentiating cells helps HPV to avoid immune detection.

The antiviral innate immune response is also a barrier that must be overcome to facilitate viral persistence (2). The detection of pathogen-associated molecular patterns (PAMPs), commonly nucleic acids, by pattern recognition receptor (PRR) and adaptor protein pairs leads to the rapid expression of type I (α, β, κ) as well as type III (λ) interferons (IFNs) (7, 8). PRR adaptor pairs include Toll-like receptors (TLR)–TRIF and retinoic acid–inducible gene-I–like receptors (RLRs)–mitochondrial antiviral-signaling protein (MAVS) as well as cyclic guanosine monophosphate–adenosine monophosphate synthase (cGAS)–stimulator of interferon genes (STING) (9–11). RLRs include retinoic acid–inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) as well as LGP2 (12). These pathways largely overlap in their use of downstream signaling components, ultimately leading to the expression of interferon-stimulated genes

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(ISGs) that can establish an antiviral state. Numerous mechanisms have been identified by which HPV blocks expression of type I IFN and ISGs, occurring largely through the actions of the E (early) 6 (E6) and E7 oncoproteins (13, 14). However, the majority of these mechanisms have been established in monolayer cultures and/or through overexpression of E6 and E7. There may be additional factors or processes that contribute to inhibition of an IFN response upon differentiation to provide an environment conducive to late viral events. In support of this, a recent study showed that the keratinocyte-mediated immune response is under the control of HPV in the upper layers of the stratified epithelium, and that the immune response differs from that observed in the basal, undifferentiated cells (15).

Recent studies identified a role for apoptotic caspases in blocking a type I IFN response in order to maintain apoptosis as an immunologically silent form of cell death (16–18). Apoptosis comprises two convergent pathways: intrinsic and extrinsic (19). Intrinsic apoptosis requires mitochondrial outer membrane permeabilization (MOMP), resulting in cytochrome *c* release and activation of caspase-9 (20). Caspase-9 then cleaves and activates the executioner caspases-3 and -7, triggering a cascade of proteolytic events that culminate in apoptosis. The extrinsic pathway is activated by the binding of specific proapoptotic ligands to their respective transmembrane receptors, resulting in the activation of caspase-8 (21). Caspase-8 either can lead directly to cleavage/activation of caspase-3 or can trigger apoptosis through the intrinsic pathway by inducing MOMP (22). In the absence of apoptotic caspase activity, viral infection or mitochondrial DNA (mtDNA) released by MOMP triggers the cytosolic cGAS–STING DNA sensing pathway, initiating type I IFN production through activation of the TBK1 (Tank-binding kinase 1)–IRF3 axis (17, 18, 23). Caspase-3 attenuates this response by cleaving cGAS as well as IRF3, which is also a substrate of caspase-8 (23, 24). Through cleavage of IRF3 as well as MAVS, apoptotic caspase activity also restrains the IFN response through the RIG-I and MDA5 pathways that are activated by RNA virus infection (23).

Previous studies demonstrated that high-risk HPV31 induces low-level activation of caspases belonging to the intrinsic apoptotic pathway upon differentiation (caspases-9, -3, and -7) (25). Treatment of HPV31-positive cells with a pancaspase inhibitor or specific inhibitors of caspase-9 or caspase-3/7 blocked productive replication, indicating a proviral role for apoptotic caspases in the viral life cycle (25). Caspase activation did not result in morphological features of apoptosis and occurred in the presence of increasing levels of antiapoptotic Bcl2, suggesting that HPV limits caspase activity by balancing the levels of pro- and antiapoptotic proteins. Additionally, the E1 viral helicase was shown to be a substrate of caspase-3/7, with caspase processing of E1 being required for efficient productive viral replication. Whether apoptotic caspases play additional roles in facilitating productive replication has not been examined. Recent studies showed that KSHV (Kaposi's sarcoma-associated herpesvirus) utilizes apoptotic caspase activity during lytic replication to block a type I IFN response (26). These results raise the possibility that HPV may also commandeer this existing function of activated caspases to prevent excessive IFN production to support productive replication and also, immune evasion.

In this study, we demonstrate that inhibition of caspase activity in differentiating HPV31-positive cells results in a significant increase in type I IFN-β as well as type III IFN-λ1 expression and secretion that can induce expression of ISGs and block productive replication in bystander cells. We show that HPV16-infected cells also exhibit caspase activation upon differentiation and require caspase activity to suppress an IFN response. Furthermore, we show that caspase-8 is required for productive replication of HPV31 and identify a critical role for caspase-8 and caspase-3 in suppressing the innate immune response induced upon differentiation. Importantly, we have found that caspase activity suppresses an IFN response triggered by the double-stranded RNA (dsRNA) sensor MDA5, indicating that immunostimulatory dsRNAs accumulate upon differentiation. Overall, these studies identify a pivotal role for apoptotic caspase activity in facilitating productive replication by regulating the innate immune response to RNA through the MDA5–MAVS pathway.

Results

Caspase Inhibition Results in a Type I and Type III IFN Response upon Differentiation of HPV31-Positive Cells. To determine if caspase activity plays a role in regulating the innate immune response during productive replication of high-risk HPVs, we utilized the CIN612 9E cell line (referred to as CIN612), which is derived from a CIN1 cervical lesion and maintains HPV31 genomes episomally (27). Previous studies demonstrated that CIN612 cells but not uninfected human foreskin keratinocytes (HFKs) exhibit caspase activation upon differentiation in high-calcium medium (25), which is sufficient to activate the productive phase of the HPV31 life cycle by 48 h. Confirmation of caspase activation in CIN612 cells is shown in *SI Appendix*, Fig. S1*A*.

For these studies, we used the US Food and Drug Administration–approved pancaspase inhibitor IDN-6556 (Emricasan), which blocks productive replication of HPV31 (Fig. 1*A*). Inhibition of cleavage of the caspase substrate poly (ADP-ribose) polymerase (PARP) was used to confirm caspase inhibition (Fig. 1*B*). Importantly, IDN-6556 treatment did not affect the ability of CIN612 cells to differentiate as shown by an increase in the differentiation-specific marker involucrin (Fig. 1*B*). We examined the effect of IDN-6556 treatment on the expression of type I *IFN-β* as well as type III *IFN-λ1*, which plays an important role in antiviral immunity at mucosal barriers (28). While differentiation of CIN612 cells alone triggered an increase in *IFN-β* and *IFN-λ1*, treatment with IDN-6556 exacerbated this response, inducing a 600- to 700-fold increase in *IFN-β*, *-λ1* expression by 72 h postdifferentiation (Fig. 1*C* and *D*). These studies suggest that caspase activation restrains excessive activation of an IFN response upon differentiation of HPV31-positive cells.

Both type I and type III IFNs induce expression of a large number of ISGs, leading to an antiviral state (29). We observed a significant increase in the expression of several ISGs, including *ISG56/IFIT1* and *ISG15* (Fig. 1*E* and *F*). Importantly, ISG56/IFIT1 has been shown to bind and shuttle the E1 viral helicase to the cytoplasm, blocking viral replication (30, 31). These results indicate that caspase inhibition results in active IFN signaling. To determine if IFN is secreted into the culture medium, we used the HEK-Blue IFN-α/β and IFN-λ reporter cells (InvivoGen). Despite an increase in *IFN-β* and *IFN-λ1* expression, IFN-α/β secretion decreased upon differentiation, with no significant changes in IFN-λ secretion (Fig. 1*G*). However, caspase inhibition resulted in a significant increase in IFN-α/β and IFN-λ secretion at 48 and 96 h postdifferentiation that was accompanied by an increase in protein levels of the ISG56/IFIT1, ISG15, and OAS2 (Fig. 1*G* and *H*). In contrast, uninfected HFKs, which exhibit undetectable levels of caspase activation upon differentiation (*SI Appendix*, Fig. S1*A*) (25), yielded no significant changes in *IFN* expression or messenger

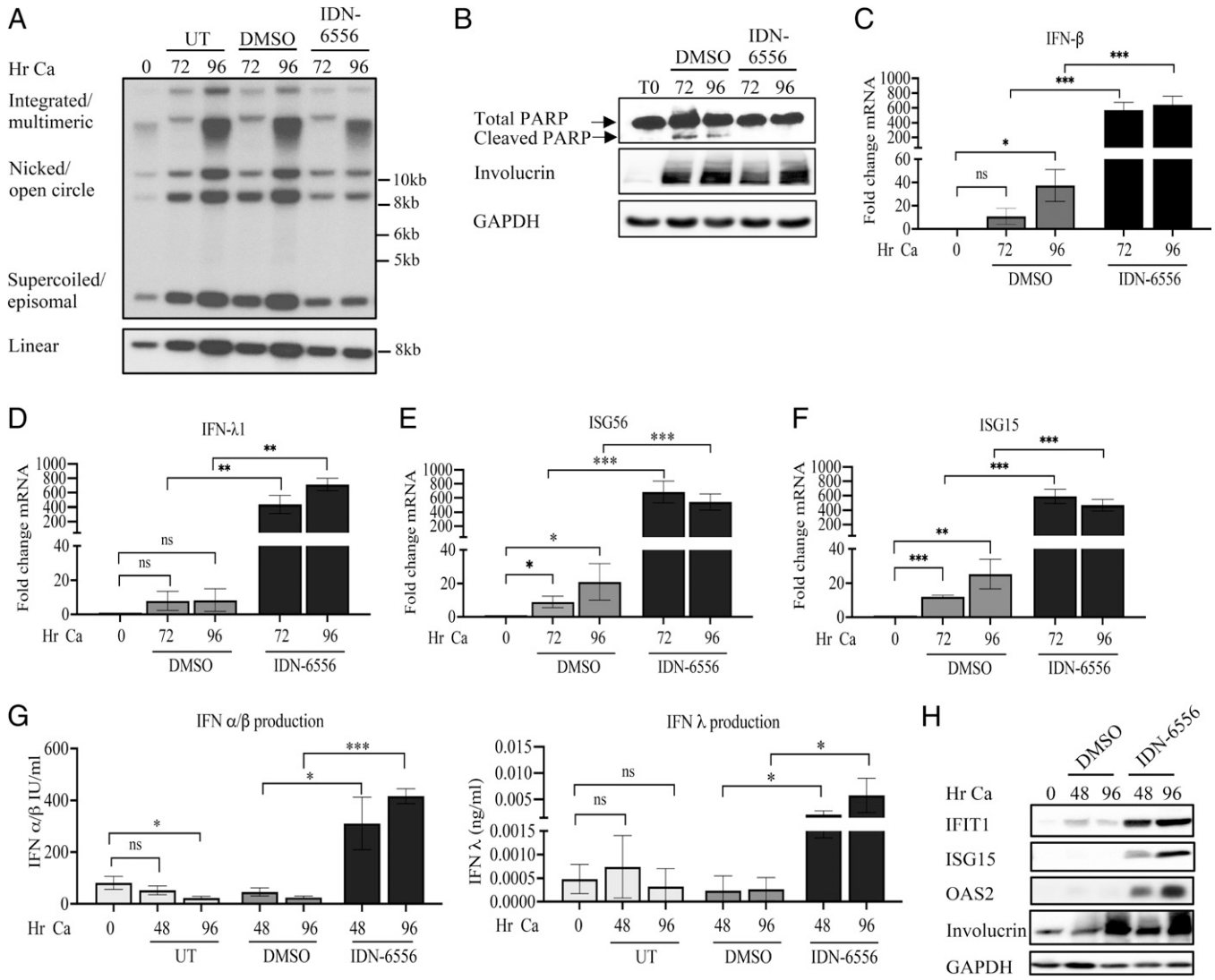

Fig. 1. Pancaspase inhibition triggers an IFN response in differentiating HPV31-positive CIN612 cells. DNA, protein, RNA, and supernatants were harvested from undifferentiated CIN612 cells (T0) or after differentiation in high calcium (72 and 96 h) in the presence of DMSO or 10 μM IDN-6556. (*A*) Southern blot analysis on DNA digested with BamHI (HPV DNA noncutter; *Upper*) or EcoRV (cuts viral DNA once; *Lower*). The HPV31 genome served as a probe. (*B*) Western blot analysis using an antibody that detects total and cleaved PARP. Involucrin is a marker for differentiation; GAPDH is a loading control. (*C–F*) qRT-PCR was performed to measure *IFN-β*, *IFN−λ1*, *ISG56*, and *ISG15* expression. Fold change was calculated using the $2^{-\Delta\Delta CT}$ method. Shown is the fold change relative to T0, which is set to one. The values represent the average of three independent experiments. Error bars represent means ± SE. Statistical significance was determined using a Student's *t* test. (*G*) IFN-α/β and IFN-λ were quantified in the supernatants of the indicated samples using the HEK Blue IFN-α/β or IFN-λ reporter cells. Shown is an average of three independent experiments. Error bars represent means ± SE. Statistical significance was determined using a Student's *t* test. (*H*) Western blot was performed for the indicated ISGs. (*A*, *B*, and *H*) Shown are representative images of at least three independent experiments. Ca, calcium; UT, untreated; Hr, hour. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$. ns, not significant.

RNA (mRNA)/protein levels of ISG56/IFIT1 or ISG15 upon IDN-6556 treatment (*SI Appendix*, Fig. S1*B–D*). Previous studies showed that HPV31 also induces caspase activation in differentiating HFKs (HFK-31 cells) (25). IDN-6556 treatment of HFK-31 cells blocked productive replication and heightened the IFN-β, IFN-λ1, and ISG response upon differentiation, similar to that observed in cervical CIN612 cells (*SI Appendix*, Fig. S2*A–D*). Inhibition of PARP cleavage was used to confirm caspase activation/inhibition (*SI Appendix*, Fig. S2*D*).

To determine if caspase activation and suppression of an IFN response are conserved across high-risk types, we used CIN1-derived W12 clone 20863 (W12E) cells, which maintain HPV16 episomally and support differentiation (32, 33). Previous studies showed that W12E cells express genes involved in a type I IFN response upon calcium-induced differentiation (15). As shown in *SI Appendix*, Fig. S2*E–H*, differentiation of W12E cells also resulted in caspase activation, as measured by cleavage of caspase-7 and PARP. Additionally, W12E cells exhibited an increase in *IFN-β* and *IFN-λ1* upon differentiation that was exacerbated by IDN-6556 treatment (*SI Appendix*, Fig. S2*E* and *F*). Furthermore, IDN-6556 treatment resulted in a substantial increase in the mRNA/protein levels of ISG56/IFIT1 and ISG15 (*SI Appendix*, Fig. S2*F–H*). Overall, these results suggest that caspase activity is a conserved mechanism of high-risk HPVs to suppress an IFN response upon differentiation.

To further confirm the role of caspases in suppressing an IFN response, we tested the pancaspase inhibitor Z-VAD-FMK, which was previously shown to block productive replication of HPV31 (25), and we confirmed this using CIN612 cells in *SI Appendix*, Fig. S3*A*. Inhibition of PARP cleavage confirmed caspase inhibition (*SI Appendix*, Fig. S3*B*). Similar to IDN-6556, Z-VAD-FMK treatment of CIN612 cells induced a significant increase in *IFN-β* and *IFN-λ1* expression as well as the mRNA/

protein levels of ISG56/IFIT1 and ISG15 upon differentiation (*SI Appendix*, Fig. S3*C–E*). In contrast, increasing caspase activation in differentiating CIN612 cells through treatment with the apoptosis inducer staurosporine (34), as evidenced by increased PARP cleavage (*SI Appendix*, Fig. S3*H*), resulted in decreased mRNA levels of *IFN-β* and *IFN-λ1* as well as decreased mRNA/protein levels of ISG56/IFIT1 (*SI Appendix*, Fig. S3*F–H*). These results provide further support for the role of caspase activity in restraining excessive activation of an IFN response in differentiating HPV-infected cells.

IFN Induction upon Caspase Inhibition Coincides with a Decrease in Productive Replication and Late Viral Gene Expression. To determine the kinetics of when the IFN response is activated relative to the block in productive replication, we examined *IFN-β* and *IFN-λ1* expression as well as a subset of ISGs in CIN612 cells over an extended time course starting at 24 h postdifferentiation (*SI Appendix*, Fig. S4). We also performed Southern blot analysis to examine viral replication as well as qRT-PCR to examine viral gene expression at each of these time points. Treatment with IDN-6556 induced expression of *IFN-β* and *IFN-λ1* as well as *ISG56/IFIT1* and *ISG15* starting at 24 h postdifferentiation (*SI Appendix*, Fig. S4*A–D*). We observed a substantial increase in the protein levels of ISG56/IFIT1 and ISG15 at 24 h that corresponded with a progressive decrease in productive replication (*SI Appendix*, Fig. S4*E* and *F*). The levels of the late genes E1^E4 and L1 were also significantly decreased at 72 and 96 h postdifferentiation, respectively, with a more moderate effect observed for the early gene E6*I (*SI Appendix*, Fig. S4*G*). The decrease in viral gene expression could stem from the inability of HPV to productively replicate upon caspase inhibition, resulting in fewer templates for transcription (6). Overall, these results suggest that caspase activity is important for restraining an IFN response to allow for productive viral replication.

Conditioned Medium Induces ISG Expression and Blocks Productive Viral Replication in an IFN-Dependent Manner. Our studies indicate that the levels of secreted IFN-α/β and IFN-λ significantly increase upon treatment with IDN-6556. However, the effect of type I or type III IFNs on productive viral replication has not been examined. As shown in Fig. 2*A*, treatment of CIN612 cells with recombinant IFN-β as well as IFN-λ1, -λ2, and -λ3 resulted in a substantial increase in the protein levels of ISG56/IFIT1, OAS2, and ISG15 that corresponded with a block in productive viral replication. Importantly, IFN treatment did not block caspase activation or induction of the differentiation marker involucrin (Fig. 2*A*), supporting a direct effect of IFN on viral replication, likely through ISG production.

To determine if the secreted IFN-α/β and IFN-λ is functional to induce ISG expression and prohibit HPV productive replication in bystander cells, we collected supernatants from differentiated CIN612 cells treated with the pancaspase inhibitor (IDN-6556) or dimethyl sulfoxide (DMSO) for 96 h (Fig. 2*B*). Naive CIN612 cells were then exposed to the conditioned medium (CM) for 6 h before the addition of high-calcium medium to induce differentiation (Fig. 2*C*). CIN612 cells treated with IDN-6556 CM resulted in a strong increase in the protein levels of ISG15, ISG56/IFIT1, and OAS2 and a defect in productive replication (Fig. 2*C*). In contrast, CM from DMSO-treated cells (DMSO CM) only modestly affected the levels of the ISG proteins and did not affect productive replication (Fig. 2*C*). Pretreatment of CIN612 cells with a mixture of neutralizing antibodies to type I IFNs and the type I receptor subunit 2 prior to the addition of DMSO CM, IDN-6556 CM (Fig. 2*D* and *E*), or recombinant IFN-β1a (*SI Appendix*, Fig. S5) blocked the induction of ISG56/IFIT1 and ISG15 mRNA and protein levels upon differentiation. These results demonstrate that type I IFNs present in the cellular supernatant are largely responsible for the induction of ISGs and the block in productive replication.

Inhibition of Caspase-3 and Caspase-8 Triggers an IFN Response in Differentiating HPV31-Positive Cells. As pancaspase inhibitors, IDN-6556 and Z-VAD-FMK can block both extrinsic and intrinsic apoptosis. Inhibition of caspase-9 (the key mediator of intrinsic apoptosis) and caspase-3 blocks productive replication of HPV31 (25). We have found that caspase-8 (mediator of extrinsic and intrinsic apoptosis) cleavage (activation) increases upon differentiation of HPV31-positive cells but not HFKs (Fig. 3*A*). Inhibition of caspase-8 activity using the selective inhibitor Z-IETD-FMK blocked productive replication in a manner similar to inhibition of caspase-3/7 (Z-DEVD-FMK) (Fig. 3*B*). PARP cleavage was blocked upon differentiation after treatment with Z-IETD-FMK and Z-DEVD-FMK, confirming caspase inhibition (*SI Appendix*, Fig. S6*A*). Inhibition of caspase-8 and caspase-3 resulted in a significant increase in the expression and secretion of IFN-β and IFN-λ1 (Fig. 3*C–E*). Additionally, we observed a significant increase in the transcript levels of *ISG56/IFIT1* and *ISG15* as well a substantial increase in the protein levels (*SI Appendix*, Fig. S6*B* and *C*). Similar effects on productive replication and ISG induction were observed when validated small interfering RNAs (siRNAs) were used to transiently knock down caspase-8 and caspase-3 expression (*SI Appendix*, Fig. S6*D–F*) (26). These results demonstrate that caspase-8 and caspase-3 play pivotal roles in preventing excessive IFN induction during the productive phase of the viral life cycle.

The IFN Response Is Induced via the TBK1–IRF3 Signaling Pathway. To determine the mechanism by which the IFN response is activated upon caspase inhibition, we monitored the activation and autophosphorylation of TBK1 (Ser172) and the phosphorylation of its downstream target IRF3, which are key early events downstream of the cGAS–STING DNA sensing pathway as well as the RIG-I–MDA5–MAVS RNA sensing pathways (Fig. 4*A*) (35). TBK1-dependent phosphorylation of IRF3 leads to IRF3 dimerization and translocation to the nucleus, resulting in transcription of type I and type III IFN genes (36). Pancaspase inhibition as well as caspase-3 and caspase-8 inhibition upon differentiation resulted in increased phosphorylation of TBK1 (Ser172) as well as IRF3 on Ser396 and Ser386, which is essential for IRF3 dimerization (Fig. 4*B* and *C*) (36). In contrast, CIN612 cells that were untreated or treated with DMSO exhibited a minimal increase in pTBK1 and pIRF3 upon differentiation. Similar effects on pTBK1 and pIRF3 were observed upon treatment with the pancaspase inhibitor Z-VAD-FMK as well as depletion of caspase-8 or caspase-3 using siRNAs (*SI Appendix*, Fig. S7). Using a small molecule inhibitor of TBK1/IKKε activity (MRT67307), we found that pancaspase inhibitor treatment in combination with TBK1 inhibition resulted in decreased levels of pIRF3 as well as a significant reduction in IFN-β and IFN-λ1 expression and secretion from CIN612 cells (Fig. 4*D–F*). Additionally, we observed decreased protein levels of ISG56/IFIT1, ISG15, and OAS2 (Fig. 4*D*). Overall, these results indicate that the IFN response is activated in a TBK1-dependent manner.

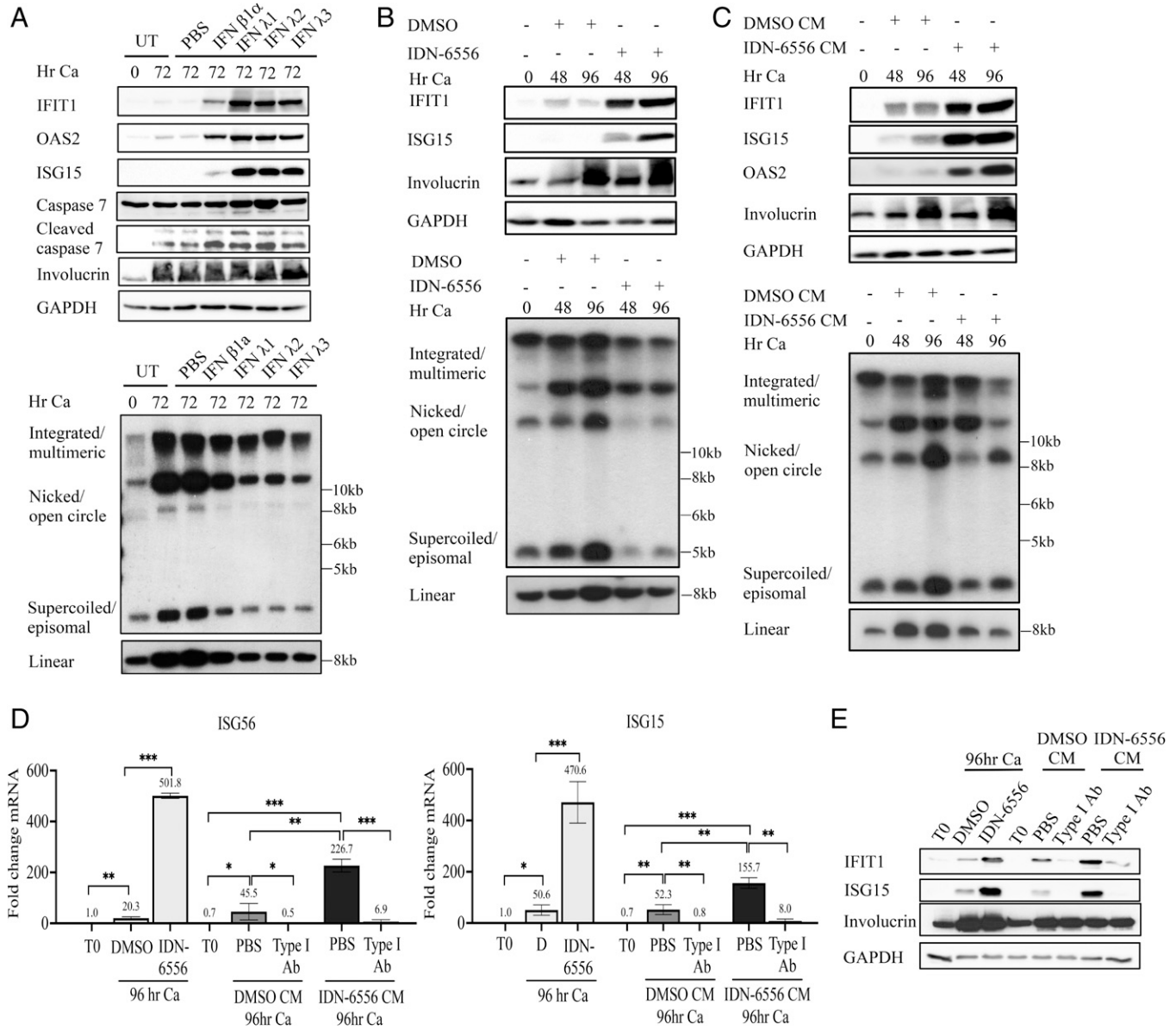

Fig. 2. Caspase inhibition results in IFN secretion that blocks productive replication. (*A*) CIN612 cells were left untreated (UT) or treated prior to differentiation for 6 h with phosphate-buffered saline (PBS), 25 IU/mL IFN-β, or 100 ng/mL IFN-λ1, -λ2, or -λ3. Cells were then differentiated for 72 h in high-calcium medium. (*Upper*) Western blot analysis was performed using the indicated antibodies. Involucrin is a differentiation control, and GAPDH is a loading control. (*Lower*) Southern blot was performed on DNA digested with BamH1 (top panel; viral DNA noncutter) or EcoRV (lower panel; cuts viral genome once). The HPV31 genome was used as a probe. (*B*) Western blot and Southern blot analyses of CIN612 cells that were undifferentiated (T0) or differentiated in the presence of DMSO or 10 μM IDN-6556 (48 and 96 h). (*C*) CIN612 cells were incubated with supernatants from *B* (DMSO CM or IDN-6556 CM harvested 96 h postdifferentiation) for 6 h prior to differentiation in high-calcium medium (48 and 96 h). Western blot analysis and southern blot analysis were then performed. (*D* and *E*) CIN612 cells were harvested as undifferentiated (T0) or differentiated in high-calcium medium for 96 h with DMSO or 10 μM IDN-6556 (lanes 1 to 3); 96 h postdifferentiation, CM was removed from the DMSO- or IDN-6556–treated plates and added to CIN612 cells that had been pretreated with PBS or the human type 1 neutralizing antibody mixture for 6 h, followed by differentiation in high-calcium medium for 96 h. (*D*) qRT-PCR was performed to measure *ISG56* and *ISG15*. Fold change was calculated using the $2^{-\Delta\Delta CT}$ method. Shown is the fold change relative to CIN612 T0, which is set to one. The values represent the average of at least three independent experiments. Error bars represent means ± SE. Statistical significance was determined using a Student's *t* test. (*E*) Western blot analysis was performed using the indicated antibodies. Involucrin is a differentiation control. GAPDH is a loading control. (*A–C* and *E)* Shown are representative images of three or more independent experiments. Ca, calcium; Hr, hour; Ab, antibody. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

The cGAS–STING Pathway Is Dispensable for the IFN Response Induced upon Differentiation. MOMP results in the release of mtDNA that in the absence of apoptotic caspase activity, can stimulate the IFN response through the cGAS–STING pathway (17, 18, 23). Upon binding to DNA, cGAS catalyzes the synthesis of cyclic GMP-AMP (cyclic guanosine monophosphate–adenosine monophosphate) that activates STING (10). STING then recruits and activates TBK1, which phosphorylates STING (Ser366) to provide a docking site for IRF3 (37). We found that pancaspase inhibition induced STING phosphorylation in CIN612 cells but not until 72 h postdifferentiation (Fig. 5*A*). Using validated siRNAs to transiently knock down cGAS and STING (Fig. 5*B*) (38), we found no effect on the phosphorylation of TBK1 or IRF3 (S386) induced by pancaspase inhibition (Fig. 5*C*), nor did cGAS–STING knockdown attenuate the enhanced expression of *IFN-β* and *IFN-λ1* or *ISG56/IFIT1* and *ISG15* (Fig. 5*D* and *E*). Rather, STING depletion resulted in an increase in the expression of *IFN-β* and *IFN-*λ1 as well as *ISG56/IFIT1* and *ISG15*, although this was not evident at the protein level (Fig. 5*D–F*). Moreover, cGAS–STING knockdown did not affect the ability of

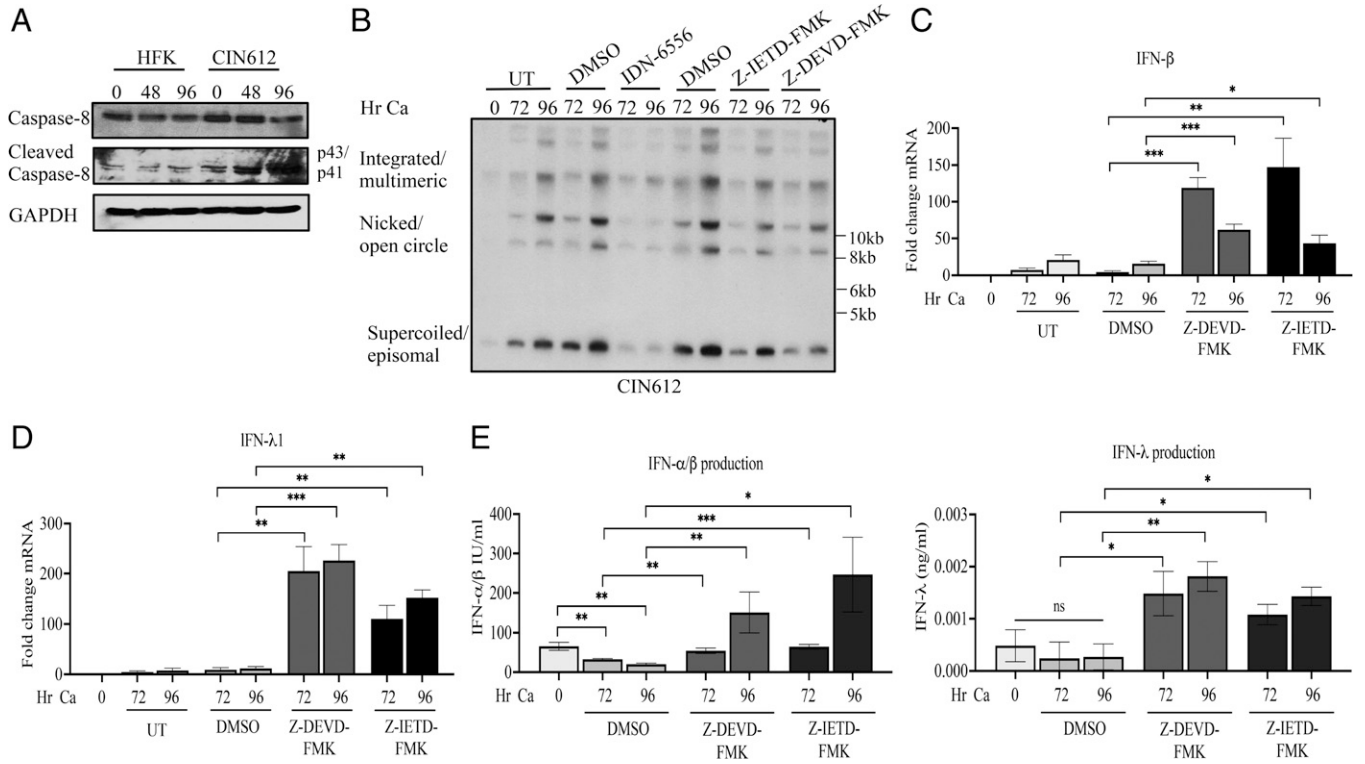

Fig. 3. Inhibition of caspase-3 and caspase-8 induces an IFN response in differentiating HPV31-positive cells. (*A*) Western blot analysis was performed for total caspase-8 and cleaved caspase-8 (p43/p41) on lysates harvested from undifferentiated HFKs and CIN612 cells (T0) or after differentiation in high-calcium medium (48 and 96 h). GAPDH is a loading control. (*B–E*) DNA, RNA, and supernatants were harvested from undifferentiated CIN612 cells (T0) or after differentiation (72 and 96 h) in the presence of DMSO, 10 μM IDN-6556, or 50 μM caspase-8 inhibitor (Z-IETD-FMK) or caspase-3 inhibitor (Z-DEVD-FMK) or left untreated (UT). (*B*) Southern blot analysis was performed on DNA digested with BamHI (HPV31 DNA noncutter). The HPV31 genome served as a probe. (*C* and *D*) qRT-PCR was performed to measure *IFN-β* and *IFN-λ1* expression. Shown is the fold change relative to T0, which is set to one. The values represent the average of at least three independent experiments. Error bars represent means ± SE. Statistical significance was determined using a Student's *t* test. (*E*) IFN-α/β and IFN-λ were quantified in the supernatants of the indicated samples using the HEK Blue IFN-α/β or IFN-λ reporter cells. Shown is an average of three independent experiments. Error bars represent means ± SE. Statistical significance was determined using a Student's *t* test. (*A* and *B*) Shown are representative images of three independent experiments. Ca, calcium; Hr, hour. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$.

HPV to productively replicate (Fig. 5*G*). Overall, these results indicate that cGAS–STING signaling is not responsible for the induction of the IFN response.

Depletion of the RLR Adapter MAVS Attenuates the IFN Response in Differentiating HPV31-Positive Cells. In addition to the cGAS–STING pathway, caspase-3 can block activation of the RLR–MAVS pathway through cleavage of MAVS and IRF3 (23). IRF3 is also a substrate of caspase-8 (24). To determine if the RLR pathways are involved in the IFN response, we used a validated siRNA to MAVS (39), which is the common downstream adaptor for RIG-I and MDA5 and has also been shown to be important in sensing other DNA viruses (40–44). We observed that MAVS knockdown in IDN-6556–treated CIN612 cells resulted in a substantial decrease in the levels of pTBK1 and pIRF3 (S386) as well as a significant reduction in expression of *IFN-β* and *IFN-λ1* (Fig. 6*A* and *B*). Additionally, the mRNA and protein levels of ISG56/IFIT1 and ISG15 were strongly reduced (Fig. 6*C* and *D*). We also found that MAVS knockdown resulted in a decrease in the basal levels of ISG56/IFIT1 and ISG15 mRNA and protein in the untreated and DMSO controls (Fig. 6*C* and *D*). Interestingly, depletion of MAVS resulted in a substantial increase in viral episomes upon differentiation in DMSO-treated cells as well as cells treated with IDN-6556 compared with the negative control (NC) siRNA (Fig. 6*E*), indicating that MAVS serves as a restriction factor for productive viral replication. The increase in viral copy number was accompanied by a significant increase in the late genes E1^E4 and L1 (*SI Appendix*, Fig. S8*A*). While we also observed an increase in the higher–molecular weight integrated/multimeric forms of the viral DNA, whether this reflects an increase in integration is currently unclear. Overall, these results indicate that MAVS serves as a restriction factor for productive viral replication and suggest that apoptotic caspase activity attenuates activation of an IFN response through the RNA-sensing RLR–MAVS pathway.

The dsRNA Sensor MDA5 Is Required for the Enhanced IFN Response Induced by Caspase Inhibition. MDA5 and RIG-I recognize distinct forms of dsRNA to stimulate the IFN response, and they are also ISGs (45–49). Indeed, caspase inhibition resulted in substantial increase the mRNA and protein levels of MDA5 and RIG-I (*SI Appendix*, Fig. S8*B* and *C*). Using validated siRNAs to transiently knock down RIG-I and MDA5 individually as well as in combination (39), we found that RIG-I silencing had a minimal effect on the induction of the IFN response upon caspase inhibition (Fig. 7*A–D*). However, MDA5 depletion resulted in a significant decrease in RLR signaling, exhibited by a decrease in pTBK1 and pIRF3 (S386) (Fig. 7*A*) and a significant decrease in the expression of *IFN-β*, *IFN-λ1*, and the ISGs *ISG56/IFIT1* and *ISG15* as well as a substantial decrease in ISG56/IFIT1 and ISG15 protein levels (Fig. 7*B–D*). Additionally, MDA5 depletion, and to a lesser extent RIG-I, resulted in decreased expression and protein levels of ISG56/IFIT1 and ISG15 induced by differentiation (DMSO) compared with the CIN612-NC siRNA DMSO-

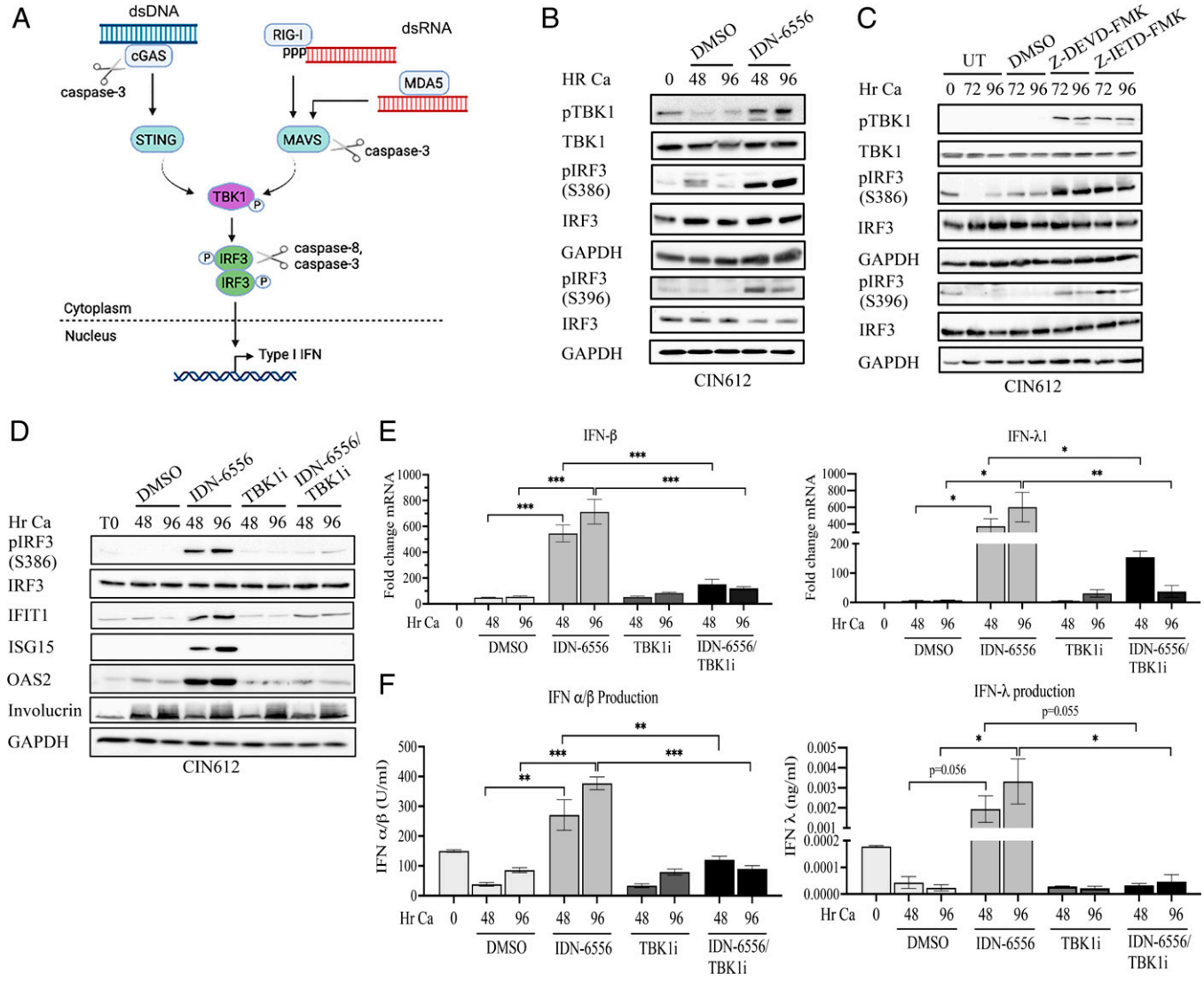

Fig. 4. The IFN response occurs in a TBK1-dependent manner. (*A*) In the cytosol, cGAS can detect host DNA as well as viral or bacterial DNA. Activated cGAS signals through its adapter STING to activate the kinase TBK1, which then phosphorylates IRF3. IRF3 dimerizes and enters the nucleus to stimulate expression of type I IFNs. RIG-I detects relatively short dsRNAs containing a 5′-tri– or diphosphate end, while MDA5 binds to long dsRNA. RIG-I–MDA5 signals through their adaptor MAVS, which then activates the TBK1–IRF3 pathway to induce IFN production. (*B* and *C*) Western blot was performed using the indicated antibodies on lysates harvested from undifferentiated CIN612 cells (T0) or after differentiation in the presence of (*B*) DMSO or 10 μM IDN-6556 and (*C*) DMSO, 50 μM Z-DEVD-FMK, or 50 μM Z-IETD-FMK for the indicated time points. (*D–F*) Protein, RNA, and supernatants were harvested from CIN612 cells that were undifferentiated (T0) or differentiated in the presence of DMSO, 10 μM IDN-6556, 2 μM TBK1 inhibitor (TBK1i; MRT67307), or IDN-6556/TBK1i for 48 and 96 h. (*D*) Western blot analysis was performed for pIRF3 (S386) as well as the indicated ISGs. Involucrin served as a differentiation control, and GAPDH served as a loading control. (*E*) qRT-PCR was performed to measure *IFN-β* and *IFN-λ1* expression. Shown is the fold change relative to T0, which is set to one. The values represent the average of at least three independent experiments. Error bars represent means ± SE. Statistical significance was determined using a Student's *t* test. (*B–D*) Shown are representative images of three independent experiments. (*F*) Supernatants were harvested from CIN612 cells that were undifferentiated (T0) or differentiated in the presence of DMSO, 10 μM IDN-6556, 2 μM TBK1i (MRT67307), or IDN-6556/TBK1i for 48 and 96 h. IFN-α/β and IFN-λ were quantified in the supernatants of the indicated samples using the HEK Blue IFN-α/β or IFN-λ reporter cells. Shown is an average of three independent experiments. Error bars represent means ± SE. Statistical significance was determined using a Student's *t* test. Ca, calcium; UT, untreated; Hr, hour. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$.

treated cells. This decrease correlated with an increase in productive replication as well as higher–molecular weight forms of viral DNA (Fig. 7*E*), similar to that observed upon knockdown of MAVS. Additionally, MDA5 depletion resulted in increased expression of the late genes E1^E4 and L1 (*SI Appendix*, Fig. S8*D*). Although MDA5 depletion did not completely rescue productive replication under conditions of caspase inhibition, this is likely attributed to the residual IFN response as well as the requirement of caspase cleavage of the E1 helicase for efficient productive replication (25). These results not only identify MDA5 as a restriction factor for productive replication of HPV31, but they also indicate that apoptotic caspases are critical in restraining the induction of an MDA5/MAVS response to inflammatory RNAs upon differentiation.

Discussion

Differentiation of HPV31-positive cells results in low-level activation of caspases belonging to the intrinsic apoptotic pathway (caspase-9, -3, -7), indicating that MOMP occurs upon differentiation (25). MOMP results in the release of mtDNA that can be sensed by the cGAS–STING pathway, resulting in the induction of a type I IFN response in the absence of apoptotic caspase activity (17, 18). Although we observed an increase

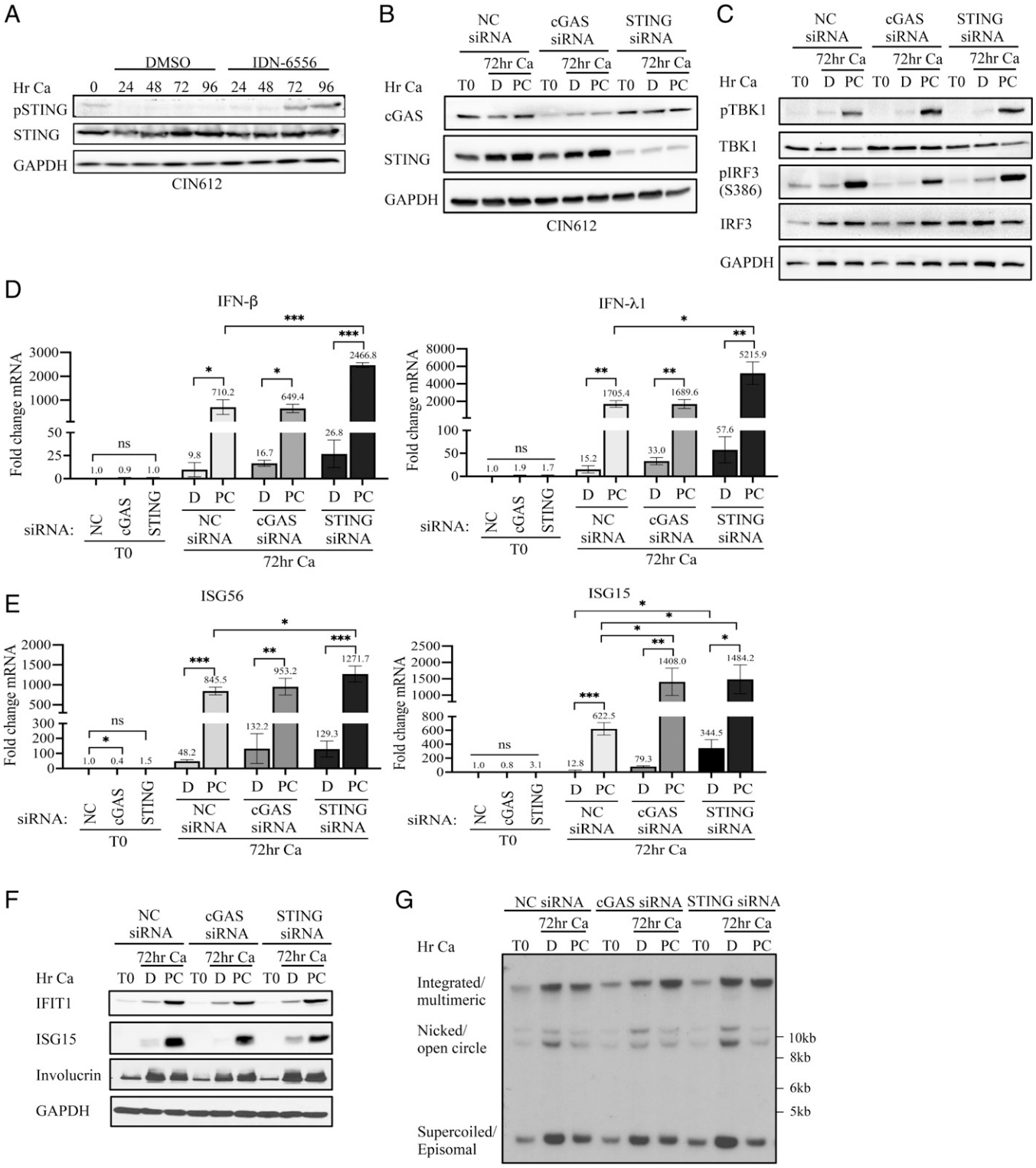

Fig. 5. The IFN response driven by caspase inhibition does not require cGAS or STING. (*A*) Western blot analysis was performed using the indicated antibodies on lysates harvested from undifferentiated CIN612 cells (T0) or after differentiation in high-calcium medium (24, 48, 72, and 96 h) with DMSO or 10 μM IDN-6556. (*B–G*) CIN612 cells were transiently transfected with a control siRNA (NC siRNA) or siRNAs specific to cGAS or STING for 48 h. Cells were then harvested as an undifferentiated sample (T0) or differentiated in high-calcium medium (72 h) in the presence of DMSO (D) or 10 μM IDN-6556 (pancaspase inhibitor [PC]). (*B* and *C*) Western blot analysis was performed using antibodies to (*B*) cGAS and STING to confirm knockdown and (*C*) pTBK1, TBK1, pIRF3 (S386), and IRF3. GAPDH is a loading control. (*D* and *E*) qRT-PCR was performed to measure *IFN-β*, *IFN-λ1*, *ISG56*, and *ISG15* expression. Shown is the fold change relative to NC siRNA T0, which is set to one. The values represent the average of at least three independent experiments. Error bars represent means ± SE. Statistical significance was determined using a Student's *t* test. (*F*) Western blot analysis was performed using antibodies to the indicated ISGs. Involucrin is a differentiation control. GAPDH is a loading control. (*G*) Southern blot analysis was performed using BamHI-digested DNA (viral DNA noncutter). The HPV31 genome was used as a probe. (*A–C*, *F*, and *G)* Shown are representative images of three or more independent experiments. Ca, calcium. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

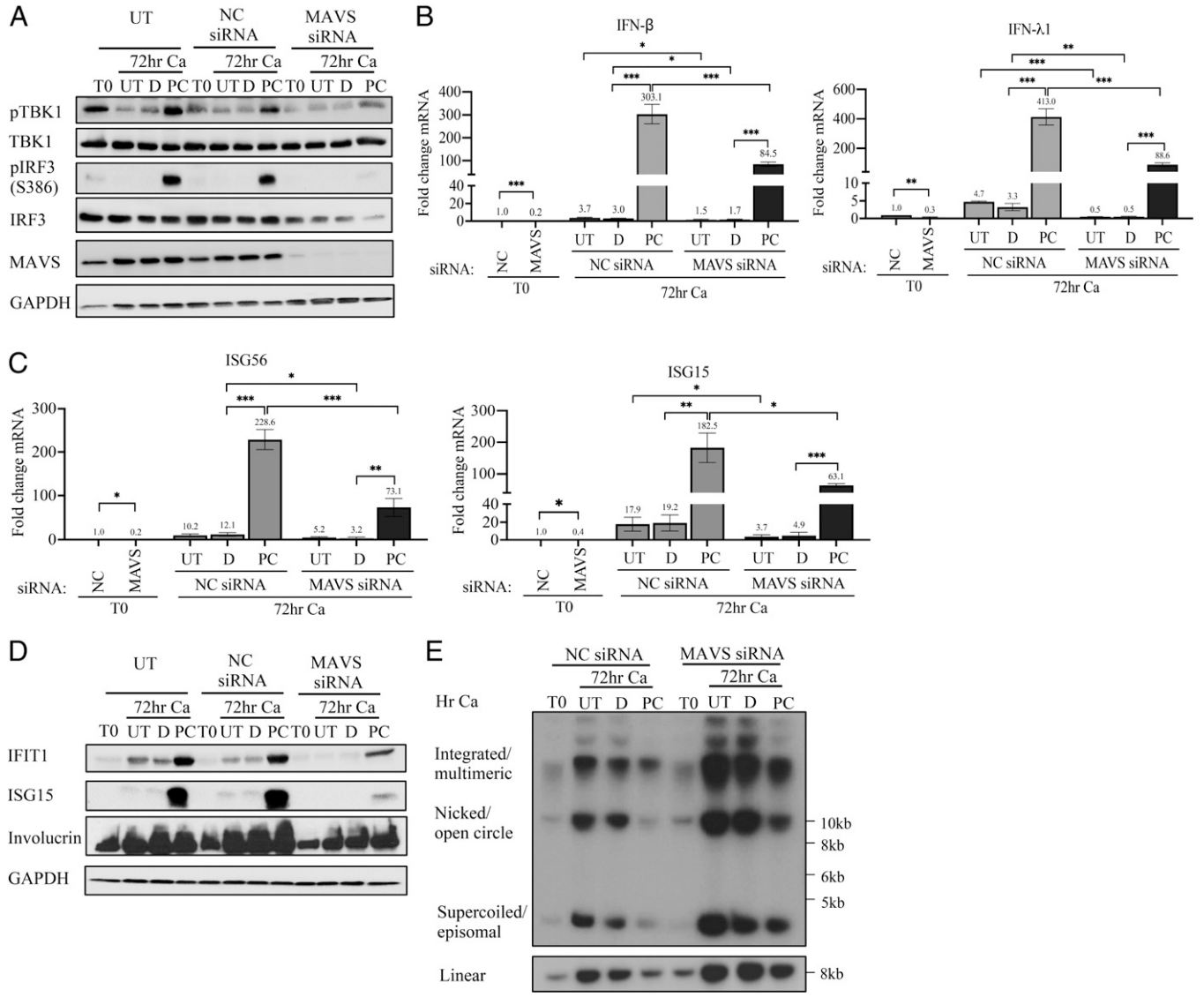

Fig. 6. MAVS is required for the IFN response induced by caspase inhibition. CIN612 cells were left untreated (UT) or transfected with control siRNA (NC siRNA) or siRNA specific for MAVS for 48 h. Cells were then harvested as an undifferentiated sample (T0) or differentiated in high-calcium medium for 72 h in the presence of DMSO (D) or 10 μM IDN-6556 (pancaspase inhibitor [PC]). (*A*) Western blot analysis was performed using the indicated antibodies. (*B* and *C*) qRT-PCR was performed to measure *IFN-β*, *IFN-λ1*, *ISG56*, and *ISG15* expression. Shown is the fold change relative to NC siRNA T0, which is set to one. The values represent the average of at least three independent experiments. Error bars represent means ± SE. Statistical significance was determined using a Student's *t* test. (*D*) Western blot analysis was performed using the indicated antibodies. (*E*) Southern blot analysis was performed on DNA digested with BamHI (*Upper*; viral DNA noncutter) or EcoRV (*Lower*; cuts viral DNA once) using the HPV31 genome as a probe. (*A*, *D*, and *E*) Shown are representative images of three independent experiments. Ca, calcium; Hr, hour. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

in pSTING upon pancaspase inhibition, we found that the cGAS–STING pathway does not contribute to the enhanced IFN response. Rather, the depletion of MDA5 or MAVS strongly reduced the IFN response triggered by caspase inhibition, with a minimal effect observed upon RIG-I depletion. Additionally, under conditions of caspase inhibition as well as caspase activation, depletion of MDA5 or MAVS increased productive replication, corresponding with an increase in expression of the late genes E1^E4 and L1 upon differentiation. Overall, these results indicate that apoptotic caspases suppress an MDA5–MAVS-driven antiviral response to dsRNA upon differentiation, preventing excessive IFN production to allow for productive replication.

Although MDA5 and RIG-I are most well known for their responses to RNA viruses, several DNA viruses have been shown to activate RIG-I and/or MDA5 as well as to encode mechanisms to block their activation (40, 50–53). The RIG-I–MAVS pathway can elicit an antiviral immune response upon de novo infection with HPV16, and the E6 oncoprotein is important for disrupting this activity (54). However, to our knowledge, whether RIG-I and/or MDA5 play a role during other phases of the viral life cycle has not been examined. Several recent studies have provided insight into how RLRs are activated during DNA virus infection, particularly for RIG-I (55). RIG-I can restrict DNA virus infection in an RNA polymerase III (RNA pol III)–dependent and –independent manner (56, 57). Cytoplasmic RNA pol III can bind and transcribe AT-rich viral DNA, generating dsRNA containing a short 5′-pppRNA (triphosphorylated RNA) that is recognized by RIG-I (57, 58). The Epstein–Barr virus (EBV)–encoded RNAs as well as adenovirus-associated RNAs are transcribed in an RNA pol III–dependent manner and induce type I IFNs by activating RIG-I (51, 52, 58).

Other studies have shown that cellular RNAs serve as a prominent PAMP to activate RLR–MAVS signaling (55). MDA5 and RIG-I both restrict lytic replication of KSHV,

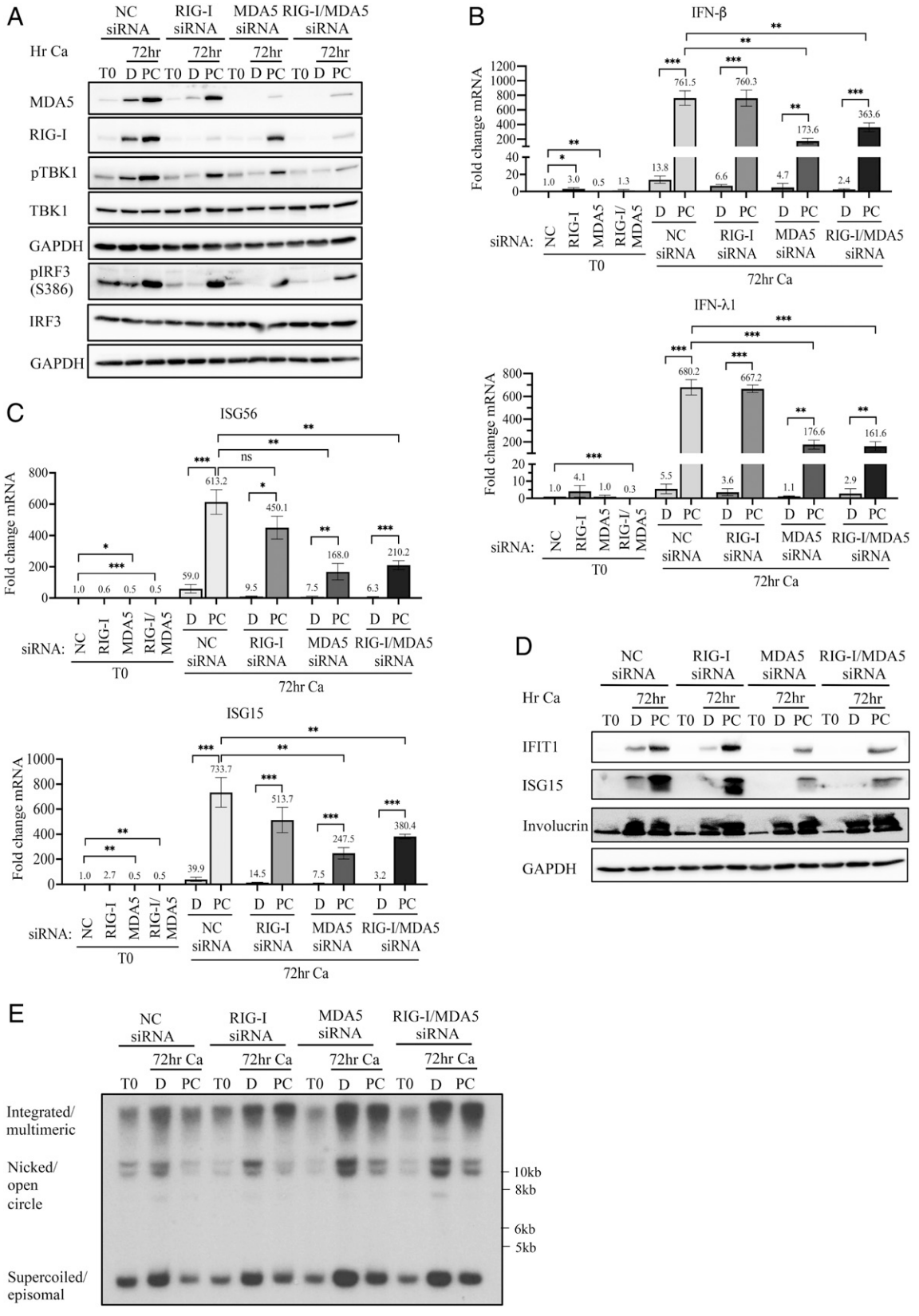

Fig. 7. The IFN response induced by caspase inhibition is driven in an MDA5-dependent manner. CIN612 cells were transfected with control siRNA (NC siRNA) or siRNA specific for RIG-I or MDA5 individually or in combination for 48 h. Cells were then harvested as an undifferentiated sample (T0) or differentiated in high-calcium medium for 72 h in the presence of DMSO (D) or 10 μM IDN-6556 (pancaspase inhibitor [PC]). (*A*) Western blot analysis was performed using the indicated antibodies, with GAPDH as a loading control. (*B* and *C*) qRT-PCR was performed to measure *IFN-β*, *IFN-λ1*, *ISG56*, and *ISG15* expression. Fold change was calculated using the $2^{-\Delta\Delta CT}$ method. Shown is the fold change relative to NC siRNA T0, which is set to one. The values represent the average of at least three independent experiments. Error bars represent means ± SE. Statistical significance was determined using a Student's *t* test. (*D*) Western blot analysis was performed using the indicated antibodies, with involucrin as a differentiation control and GAPDH as a loading control. (*E*) Southern blot analysis was performed on DNA digested with BamHI (viral DNA noncutter). The HPV31 genome was used as a probe. (*A*, *D*, and *E*) Shown are representative images of three independent experiments. Ca, calcium; UT, untreated; Hr, hour. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

with misprocessed cellular RNAs representing the bulk of the RIG-I and MDA5 substrates (40, 59). Additionally, RNA pol III–directed expression of pseudogene RNAs during Herpes simplex virus (HSV), EBV, and KSHV infection can be sensed by RIG-I (60). While the host ligands of MDA5 are less well defined, MDA5 can be activated under conditions in which mitochondrial RNA degradation is compromised, resulting in the escape of mitochondrial dsRNA into the cytoplasm (61). Additionally, inhibition of DNA methylation induces the expression of endogenous retroviral elements that can activate the MDA5–MAVS recognition pathway (62, 63). Future studies will need to determine the molecular details of how MDA5 is activated in differentiating HPV-positive cells and the identification of the RNA species that are bound to MDA5 upon caspase inhibition.

Our results indicate that caspase-8 and caspase-3 are critical to suppress the IFN response induced upon differentiation. Apoptotic caspases, particularly caspase-3, prevent excessive IFN production through the cleavage of multiple key proteins, including cGAS in cytosolic DNA sensing and MAVS in cytosolic RNA sensing (23). Additionally, IRF3, a common downstream component of both pathways, can be cleaved by caspase-3 and caspase-8 (23, 24). Caspase-8 also prevents excessive activation of IFN pathways through direct interaction with components of the RIG-I signaling complex (64). Our finding that pancaspase inhibition as well as depletion/inhibition of caspase-8 or caspase-3 results in an increase in pTBK1 suggests that caspase activity attenuates MDA5–MAVS signaling upstream of TBK1 phosphorylation, possibly through cleavage of MAVS. Additionally, the observation that STING is phosphorylated upon caspase inhibition suggests that apoptotic caspases also attenuate this pathway, possibly through cleavage of cGAS.

Our finding that inhibition/depletion of caspase-8 results in an IFN response that is similar to that observed upon inhibition/depletion of caspase-3 suggests that they act in a linear pathway, with caspase-8 activity leading to processing and activation of caspase-3. Caspase-8 can activate the intrinsic pathway through cleavage of Bid to tBid, which translocates to the mitochondria and activates Bax/Bak to induce MOMP (22). Determining if caspase-8 induces MOMP upon differentiation as well as identifying the relevant substrates of caspase-3 and caspase-8 in differentiating cells will provide insight into how these caspases restrain the IFN response.

The HPV E6 and E7 oncoproteins have evolved a multifaceted approach to avoid immune surveillance by targeting multiple innate immune effectors, including the cGAS–STING and RIG-I pathways (13, 54, 65–69). Previous studies showed that expression of HPV31 E6 or E7 alone is sufficient to induce activation of caspases-9, -3, and -7 upon differentiation (25). Interestingly, several types of HPV E6 proteins can bind and activate caspase-8 without resulting in increased apoptosis (70–72). E6 proteins have also been shown to alter the subcellular distribution of caspase-8, leading to its accumulation in the nucleus (72). Caspase-8 is typically cytoplasmic, raising the intriguing possibility that E6-induced nuclear localization exposes a new set of substrates to caspase activity. Whether E6 and/or E7 expression is sufficient to activate caspase-8 upon differentiation is currently unknown. Determining if E6/E7-induced caspase activity is sufficient to block an IFN response upon differentiation and if the IFN response is triggered in a manner similar to that observed in the context of a viral infection are important areas of future research. In addition to E6 and E7, E1 and E2 have also been reported to block expression of IFN and ISGs in keratinocytes when overexpressed (73, 74). Additionally, E5 has been shown to repress IFN signaling in the context of HPV16-infected keratinocytes (75). How HPV proteins work together to regulate the innate immune response during the viral life cycle will be an interesting area of future investigation.

Several viruses, including EBV and KSHV, hijack caspase activity to facilitate viral replication (76). Similar to HPV, KSHV commandeers caspase-8 activity to suppress a type I IFN response during lytic replication (26). EBV also uses caspase activity to cleave and inactivate cellular proteins that serve as restriction factors for lytic replication, including PIAS1 and components of the N6-methyladenosine modification machinery (77, 78). Additionally, caspase activity processes and activates EBV proteins required for viral replication (79). Similarly, HPV31 uses caspase activity to cleave the viral helicase E1, which is required for efficient productive replication (25). The caspase-3/7 cleavage site in E1 (^{46}DxxD^{49}) is conserved in all genital HPVs, suggesting that caspase activity may be a conserved mechanism by which HPVs establish a replication competent environment upon differentiation. ISG56/IFIT1 has been shown to relocalize E1 from the nucleus to the cytoplasm, blocking viral replication (30, 31). Apoptotic caspase activity is likely critical to maintain ISG56/IFIT1 at basal levels to ensure nuclear localization of E1 during productive replication. Caspase activity likely contributes to productive replication through cleavage of multiple pro- and antiviral substrates. The discovery of additional caspase targets will provide further insight into the regulation of productive viral replication.

Our data clearly show that apoptotic caspases control innate immunity during productive viral replication of HPV31 by attenuating MDA5–MAVS activation. Our finding that caspase activation also suppresses an excessive IFN response in HPV16-infected cells suggests that high-risk HPVs may use a common strategy for immune evasion upon differentiation. Future studies will focus on determining if MDA5 and MAVS also serve as restriction factors for productive replication of other high-risk types. Importantly, the ability of high-risk HPVs to suppress an IFN response upon differentiation has implications for other aspects of the viral life cycle. Previous studies showed that IFN treatment of high-risk HPV–positive cells results in episomal loss and outgrowth of cells containing integrated viral genomes (80–82). Viral genome integration is a dead end for the virus, resulting in high levels of E6 and E7 that under certain circumstances can fuel carcinogenesis (83). Our studies show that caspase inhibition significantly increases IFN secretion from differentiating cells. Hijacking of apoptotic caspase activity to suppress IFN production in differentiating cells may impede episomal loss in the proliferating basal cells, allowing for completion of the viral life cycle. However, this strategy could also, in turn, promote viral persistence, a major risk factor for cancer development.

Materials and Methods

Information on RNA extraction, qPCR, siRNA transfection, quantification of IFN using the HEK BlueTM IFN-α/β and IFN-λ reporter cells, reagents, antibodies, and statistical analysis are described in the *SI Appendix, SI Materials and Methods*. Cell culture, calcium-induced differentiation, western blot analysis and Southern blot analysis have been previously described (25, 84), and the details are presented in the *SI Appendix, SI Materials and Methods*.

Data Availability. All study data are included in the article and/or *SI Appendix*.

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