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Letter

Response to Voinnet et al.

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Coupling a promiscuous nuclease to a specific RNA-based adaptor is a common cellular antiviral strategy that can be observed in all three domains of life (tenOever, 2013). Interestingly, the universal utility of this RNA-based antiviral strategy is not observed in the domain eukaryota. For example, although the entire plant kingdom relies on virus-specific small interfering RNAs (vsiRNAs) to defend against viral pathogens, the chordate phylum of the animal kingdom, which includes all vertebrates, utilizes a protein-based strategy that is dependent on the family of type I interferons (IFN-I) (tenOever, 2013). Given the evolutionary necessity for such defense systems, it seems reasonable to postulate that a phylum such as chordates would also utilize RNAi or have at least once used it in its evolutionary past. This idea is supported by a number of experimental observations. First, chordates encode many of the necessary components for comprising an antiviral RNAi system (tenOever, 2013). Second, an inhibitor of the small RNA machinery was discovered in vaccinia virus, suggesting that small RNAs may impose some selective pressure on viruses (Backes et al., 2012). Third, two recent reports found evidence for vsiRNA fragments with RNAi-like signatures in mammalian cells (Li et al., 2013; Maillard et al., 2013). Although we would argue that none of the above observations directly demonstrated antiviral function, the collective knowledge of these findings has recently reignited the idea that mammals may evoke an antiviral RNAi response in addition to utilizing the well-known IFN-I system.

In an effort to evaluate the contribution of a mammalian RNAi system with that of IFN-I, we enabled a poorly replicating RNA virus with either the capacity to block IFN-I or RNAi and administered these viruses to wild-type mice or mice lacking IFN-I signaling capacity (Backes et al., 2014). This paper, which concluded that RNAi was not a physiological contributor to the IFN-mediated antiviral response, was recently critiqued for the choice of virus and some of its methodology by the authors who reported on the RNAi-like signatures in mammals (Voinnet and Ding, 2014). Given that there is no evidence for the evolution of a defense strategy that is virus specific, we contend that any virus could have been used for these studies so long as it did not encode a suppressor of this putative RNAi activity. Although our small RNA sequencing (RNA-seq) data could have been presented in many different ways (with regards to strand choice), it was the enrichment of small RNA reads from the ends of vesicular stomatitis virus (VSV) that prompted us to move forward with this model system, given that the genomic ends would be most vulnerable to the nonprocessive cleavage of mammalian Dicer. Given that our work is addressing what should represent a universal antiviral defense system, how the small RNA data were presented in the original publication has no bearing on the overall findings of the paper. Indeed, we show that in the absence of Dicer, the small RNAs mapping to the genomic ends remain, suggesting that, should antiviral RNAi exist at all in these cells, it is noncanonical, and therefore there is little precedent for how small RNA data from mammals should be handled. It should also be noted that the Dicer cells used in this study were clonally selected from Cre-treated, Dicer-inducible stem cells so there was no possibility of wild-type contamination. These cells were used only as a means of evaluating the biogenesis of the small virus-derived RNAs detected, and, given the fact that differentiation is impaired in the absence of microRNAs (miRNAs), we intentionally did not refer to these cells as fibroblasts or stem cells in the manuscript.

Given the lack of knowledge concerning antiviral RNAi in mammals, we chose to evaluate the contribution of small RNAs in the antiviral response by using recombinant viruses and a correlation with replication levels—the same approach used in the original studies whose bold titles declared the general identification of RNAi in mammals (Li et al., 2013; Maillard et al., 2013). To this end, we infected wild-type mice and mice lacking an IFN-I system with VSV expressing a control cassette, an antagonist to IFN-I, or VP55 (the vaccinia virus antagonist we identified that results in complete tailing of Ago-associated small RNAs [Backes et al., 2012]). We reasoned that, should mammalian antiviral RNAi exist, VP55 activity evolved to inhibit it. We were further encouraged by the fact that VSV expressing VP55 was no longer sensitive to the exogenous introduction of an siRNA. Despite this activity, we found that only the addition of the IFN-I antagonist improved virus growth and concluded that mammalian antiviral RNAi is not a significant contributor to our IFN-based antiviral defenses. Although we discuss in the paper that RNAi activity may be relevant in some cell types, our data do support the conclusion that, in vivo, VSV is not encountering Ago-associated small RNAs that are inhibiting replication, suggesting that a very large subset of cells, including monocytes and macrophages, which are the primary targets of VSV (Iannacone et al., 2010), fail to evoke an antiviral RNA response by either miRNAs or any other Ago-associated small RNAs.

With regards to VSV encoding a suppressor of RNAi silencing, we demonstrate that VSV can be inhibited by host miRNAs and by exogenously introduced siRNAs (mimicking the established antiviral RNAi response). Furthermore, we demonstrate that VSV can successfully process its own miRNA in order to conclude that the virus does not express

an antagonist of this pathway. Whereas Voinnet and Ding (2014) argue that a suppressor may exist and simply not have had the necessary time to inhibit these processes they failed to acknowledge that we also successfully prevented siRNA-mediated targeting when VSV was enabled with VP55, a known RNAi antagonist. Given that the virus-derived small RNAs detected in the original studies claiming mammalian antiviral RNAi were never shown to have silencing potential, any further means for assessing the presence or absence of a repressor will demand the capacity to first demonstrate this activity.

It should also be noted that our paper is not the only recent publication that failed to support many of the ideas put forth for RNAi in mammals. First, the idea that pluripotent cells utilize antiviral RNAi because they do not respond to IFN-I was recently found to be unsubstantiated, given that embryonic stem cells do elicit a transcription response, albeit somewhat muted, to this antiviral cytokine family (Wang et al., 2014). Furthermore, the response to virus infection has evolved to shut down small RNA silencing in mammalian cells through the ribosylation of RNA-induced silencing complex (Seo et al., 2013). In fact, our own in vivo data with VSV-VP55 (Backes et al., 2014) is in complete agreement with the results generated from Seo et al. (2013). These findings alone would make mammalian antiviral RNAi in any cell type with the capacity to respond to IFN-I seem unlikely. Lastly, in an effort to determine whether Dicer could elicit an antiviral activity as it does in other animal phyla, Bogerd et al. (2014) generated a Dicer knockout cell line and tested it against a wide range of viruses. With the exception of those viruses that generate their own miRNAs, this paper demonstrated no increased replication in the absence of the nuclease with dengue virus, West Nile virus, yellow fever virus, sindbis virus, Venezuelan equine encephalitis virus, measles virus, influenza A virus, reovirus, wild-type VSV, or human immunodeficiency virus type 1 (Bogerd et al., 2014).

Reaching a general consensus concerning the relevance of mammalian antiviral RNAi will demand contributions from many groups. Although our studies support an "IFN-I-only" hypothesis, we do not deny that this activity may still reside in certain cells that were not accessible to VSV and are genuinely incapable of responding to IFN-I. Indeed, we concluded that small RNA silencing is not a physiological contributor to the "IFN-mediated cellular response." This very topic was discussed in length in our original publication (Backes et al., 2014). In closing, these challenges are a necessary part of the scientific process, and it is clear that time and the future work of independent labs will be needed to better resolve if small RNAs do significantly contribute to the mammalian response to virus infection.

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