Paris, France; [5]APHP, Hôpitaux Universitaires Paris–Seine Saint-Denis, Site Jean Verdier, Pôle d'Activité Cancérologique Spécialisée, Service d'Hépatologie, Bondy, France; [6]CHU de Bordeaux, Department of Hepatology, Hôpital Saint-André, Bordeaux, France; [7]INSERM, UMR 1053; Université de Bordeaux, Bordeaux, France; [8]Assistance Publique-Hôpitaux de Paris, Department of Pathology, CHU Henri Mondor, Créteil, France; [9]Assistance Publique-Hôpitaux de Paris, Department of Digestive and Hepatobiliary Surgery, CHU Henri Mondor, Créteil, France; [10]INSERM, U955, Créteil, France; [11]IntegraGen, Evry, France; [12]CHU de Bordeaux, Pellegrin Hospital, Department of Pathology, Bordeaux, France; [13]Institut Gustave Roussy, Core Europe, Villejuif, France; [14]Assistance Publique-Hôpitaux de Paris, Hopital Europeen Georges Pompidou, Paris, France

Correspondence: Jessica Zucman-Rossi (jessica.zucman-rossi@inserm.fr)

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Reply to "Wild-type AAV Insertions in Hepatocellular Carcinoma Do Not Inform Debate Over Genotoxicity Risk of Vectorized AAV"

We greatly appreciate the letter by Jean-Charles Nault and colleagues[1] in response to our recent editorial[2] in *Molecular Therapy* discussing their paper, "Recurrent AAV2-Related Insertional Mutagenesis in Human Hepatocellular Carcinomas."[3] In particular, we welcome the authors' clear statement that "AAV2 insertions in spontaneous human HCC...are clearly unrelated to vectorized AAV used in gene therapy" and the open discourse on the interpretation of the authors' scientific findings with regard to wild-type (wt) AAV2 biology and cancer. We note here a few points that we think ought to be discussed further.

Were the identified wtAAV2 sequence fragments driver or passenger mutations? As Nault and colleagues point out, the development of solid tumors is a multiple-step process involving many mutations. Hence, a retrospective analysis of the sequential steps leading to tumorigenesis is challenging, and often impossible. As such, the authors' contention that the identified partial wtAAV sequences represent activating driver mutations remains unproven for us. (i) How can one rule out that a passenger wtAAV mutation became clonally expanded because it had integrated into a cell that subsequently became cancerous? In that case, how would it differ in appearance from a driver mutation? (ii) Based on the data provided in their paper,[3] 81.1% of the AAV-positive hepatocellular carcinoma (HCC) cases (9/11 biopsies) are attributable to AAV-unrelated etiologies or mutations, and some of these are suggested to be driver mutations whereas others have not been studied. (iii) Furthermore, if wtAAV sequences indeed function as driver mutations, should they not be clonal in all rather than only 7/11 of the wtAAV-positive biopsies? (iv) According to the authors' work in 2013, TERT promoter mutations are present in ~60% of HCC,[3] but, intriguingly, only one wtAAV-related TERT promoter mutation was identified among the AAV-positive HCCs. (v) Induced overexpression does not necessarily equate to driving cancer development, because—particularly following integration events—the resulting messenger RNA might not be functional. Experiments to evaluate these possibilities are lacking. Along these lines, how many AAV integrations did the authors identify in "HCC-related genes" in nontumor tissues, and did these also lead to overexpression of the affected genes? Some of these aspects may become much clearer once a complete integration analysis of both the tumor and nontumor samples is available. Nevertheless, given the small number of related events in this first report linking wtAAV sequence fragments and HCC, a statistical consideration is impossible.

The authors draw a parallel between HBV or EBV and AAV. As far as tumorigenic potential is concerned, HBV-induced HCC is not necessarily a consequence of HBV integration. Chronic HBV infection is also characterized by chronic inflammation, oxidative stress, disruption of cellular pathways, and other effects, which are all associated with increased risk of cancer. From a virology point of view, HBV and wtAAV are quite different: the former exhibits several genomic stages, active replication, a high rate of mutation, and the presence of HBV proteins affecting cellular pathways; most importantly, HBV lacks an active integrase. Furthermore, the EBV–host interaction differs substantially from that of AAV. In particular, one has to take into account that EBV (like HBV) is replication-competent, in contrast to wtAAV, which is dependent on helper viruses for replication.

The authors did not observe wtAAV integration in AAV integration site 1 (AAVS1). Numerous groundbreaking studies, although not based on high-throughput sequencing, have demonstrated the preferred integration of wtAAV2 into AAVS1—located at human chromosome 19—and have uncovered the molecular mechanism underlying this integration preference, identifying the multifunctional, nonstructural wtAAV Rep proteins and specific sequence elements shared by AAV2's inverted terminal repeats and AAVS1 (Rep-binding sites and terminal resolution sequence motifs) as key factors.[5–8] Consistent with this, several publications have shown that Rep-deficient vectors gained AAVS1 targeting upon administration of Rep protein.[9–12] As yet, only two large-scale wtAAV integration analyses performed on primary cells have been published. They differed with respect to the frequency of integration into AAVS1 that was determined (2.5% and 8.9%, respectively), but nevertheless confirmed AAVS1 as an integration hot spot.[13,14] Thus, although we acknowledge that one has to assume not only methodological variations in integration site retrieval but also cell-intrinsic factors that may bias integration site distribution of wtAAV, we do still find it quite remark-

able that Nault and co-workers did not find a single wtAAV integration in AAVS1.

To summarize, although we acknowledge Nault and colleagues' clear statement regarding the structural and functional discrimination between wtAAV and AAV vectors, we continue to challenge the suggested oncogenic role for wtAAV-derived sequences. It will be very interesting to follow future studies addressing this topic, including those revealing differences between integration events in mice and humans.[15] Given the large number of sequenced HCCs worldwide, such sequence data should shed more light on wtAAV's oncogenic or even antioncogenic role in HCC formation.[16]

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Manfred Schmidt[1] Irene Gil-Farina[1] and Hildegard Büning[2]

[1] Department of Translational Oncology, German Cancer Research Center (DKFZ), National Center for Tumor Diseases (NCT), Heidelberg, Germany; [2]Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany

Correspondence: *Hildegard Büning (buening.hildegard@mh-hannover.de)*

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