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meeting point

Everybody wins! Poland hosts thrilling competitions of viruses, RNAi and football teams

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The ESF–EMBO conference on 'Antiviral RNAi: From Molecular Biology towards Applications' took place in June 2012 in Pultusk, Poland. It brought together scientists working at the interface of RNAi and virus infections in different organisms, covering the complete range from basic mechanisms of RNA silencing to RNAi-based antiviral therapy.

Complex interactions occur between viruses and RNA silencing pathways. RNA interference (RNAi) processes viral double-stranded RNAs into viral small interfering RNAs (vsiRNAs) to guide sequence-specific cleavage of viral RNA, thereby restricting virus infection and pathogenesis. Cellular microRNAs (miRNAs) affect the viral life cycle by regulating cellular and viral protein expression. Moreover, viruses encode their own miRNAs or modulate cellular miRNA expression to regulate viral and host gene expression. Finally, RNAi might be exploited to induce disease resistance in agricultural crops and to develop new human antiviral therapies. Concurrent with the European football cup, Poland hosted another event of significant interest: the ESF–EMBO conference on 'Antiviral RNAi: From Molecular Biology towards Applications', organized by Juan Antonio Garcia (Centro Nacional de Biotecnologia, Campus de la U. Autonoma, Spain), Ben Berkhout (Academic Medical Center, U. Amsterdam, The Netherlands) and Jens Kurreck (Institut für Biotechnologie, Technische U. Berlin, Germany), which brought together scientists to discuss progress in this thriving field.

Defence and counter-defence

Since the discovery in the mid-1990s that RNAi is an important antiviral defence mechanism in plants, many labs have tried to recapitulate this finding in other species including *Caenorhabditis elegans*. Yet, the lack of a natural virus infection model has long precluded the analysis of the role of the nematode's systemic and transgenerational RNAi response in antiviral immunity. Marie-Anne Felix (Institute of Biology, École Normale Supérieure, Paris, France) presented her progress towards understanding the role of RNAi in nematode infections by natural pathogens. When comparing the sensitivity of 97 *C. elegans* isolates to the nodavirus Orsay virus, she noted a striking variation in viral RNA levels over several logs, which generally correlated well with the somatic RNAi competence of the different worm strains. However, some sensitive strains were RNAi competent and vice versa, indicating that other host factors also affect sensitivity to virus infection. Felix will now conduct genome-wide association studies to map the loci encoding these factors.

Photo courtesy of M. De Noyette

> **…the interaction between the silencing machinery and viruses is more complex than previously thought**

Several talks highlighted that the interaction between the silencing machinery and viruses is more complex than previously thought. One important consequence of silencing is that viruses are under strong selection pressure, as variants that can evade silencing have a huge advantage. This led to the evolution of virus-encoded proteins that can suppress gene silencing, as presented by József Burgyán (Agricultural Biotechnology Centre, Gödöllő, Hungary). Ronald P. van Rij and Joël van Mierlo (Radboud U. Nijmegen Medical Centre, Nijmegen, The Netherlands) analysed several natural *Drosophila* pathogens for the presence of viral RNAi-suppressive activity. They identified a potent RNAi suppressor in Nora virus that inhibits cleavage activity of a preassembled RISC complex, underlining the importance of slicing of viral target RNAs in the antiviral RNAi response.

Viral RNAi suppressors can reduce the effect of silencing that is induced by the virus itself, but not if the RNAi machinery is already primed against a virus before infection. One such scenario occurs when a host is engineered to express artificial miRNAs that target viral RNA genomes. Santiago Elena (CSIC, Valencia, Spain) found that, in plants, viruses are able to follow a strategy to evade silencing in this situation, namely, by mutating the miRNA target site within their genome. Deep sequencing of the escape mutants showed that the rapid evolution of viruses in plants expressing antiviral miRNAs followed complex dynamics involving mutation, selection and drift [1].

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Jean-Luc Imler (U. Strasbourg, France) presented a genetic and proteomic analysis of the antiviral RNAi pathway in *Drosophila*. Core antiviral RNAi components Dicer 2, R2D2 and Argonaute 2 (Ago2) were pulled down from non-infected cells and from cells infected with one of three model viruses—Drosophila C virus, flock house virus or vesicular stomatitis virus. By using mass spectrometry, Imler then collectively identified roughly 100 proteins. Strikingly, the composition of Dicer and RISC complexes differed between non-infected cells and the different virus infections, suggesting a dynamic regulation of the antiviral RNAi machinery during infection.

Curiously, viruses do not just evade this layer of defence but also exploit it. Burgyán showed that a satellite RNA-derived small RNA targets a host mRNA through RNAi and that this is important in symptom development [2]. Vitantonio Pantaleo (Istituto di Virologia Vegetale, CNR, Bari, Italy) is further pursuing this avenue of investigation through a genome-wide approach. Pantaleo generated 'degradome' libraries from virus-infected grapevine to identify host mRNA targets. These libraries contain the 5′ ends of non-capped RNAs that are potential cleavage products of RISC loaded with viral siRNAs. It will be interesting to see how truly widespread is the targeting of host mRNAs by vsiRNAs.

Viral small RNAs

One essential trend is the use of next-generation sequencing technology to identify viral and cellular small RNAs in infected cells. This approach monitors viral targeting by the RNAi machinery and provides insight into the viral Dicer substrates. Esther Schnettler (U. Glasgow Centre for Virus Research, UK) presented her work on small RNAs in arthropod-borne (arbo)virus infection of the tick *Ixodus scapularis*. She analysed a tick cell line containing a Langat virus replicon, as a model for tick-borne encephalitis virus. Unlike insect vsiRNAs that are mostly 21 nt long, Langat virus small RNAs were predominantly 22 nt and derived from both the (+) and (–) viral RNA strands, with an enrichment at the two ends of the viral genome. Van Rij profiled viral small RNAs in *Drosophila* infected with Nora virus, as well as in cell lines derived from the vector mosquitoes *Aedes aegypti* and *Aedes albopictus* infected with the Sindbis virus. In both cases, Dicer-2-dependent vsiRNAs with a typical length of 21 nt were observed, scattered across the genome and mapping in similar numbers to the viral (+) and (–) strands. Strikingly, in *Aedes* cells, an additional population of viral small RNAs showed the characteristics of ping-pong-derived piwi-associated RNAs [3]. Arboviruses replicate efficiently in both their mosquito and vertebrate hosts, which provides an excellent opportunity to study viral small RNAs from the same virus in two disparate hosts. Erika Girardi (U. Strasbourg, France) analysed the Sindbis virus small RNA profile in two mammalian cell lines. In contrast to their distribution in insects, about 99% of the viral small RNAs mapped to the viral (+) strand and did not show a strong enrichment for a 21 nt size, whilst small RNAs mapping to the viral (–) strand were mostly 22 nt. Their Dicer dependence and importance for viral infection are under investigation.

By using next-generation sequencing technology, several studies identified 'hot spots' in viral genomes that produce many more small RNAs than other regions. However, Tamas Dalmay (U. East Anglia, Norwich, UK) presented new data that revealed a bias during library preparation [4]. Deep sequencing of libraries made with completely degenerated oligoribonucleotides showed that some RNA sequences were over- or under-represented, and that 58% of the possible sequences were not captured at all. The main source of the bias was found to be the RNA ligation steps: small RNAs with stable structures ligated more efficiently to the adaptors and in turn produced higher read numbers. On the basis of this result, four degenerate nucleotides were added to the ends of the commercial Illumina adaptors to form stable structures with a larger diversity of small RNAs. Indeed, libraries prepared with the new adaptors produced similar read numbers for most sequences in the degenerate pool. These results indicate that small RNA profiles generated by Illumina adaptors should generally be interpreted with caution and that 'hot spots' observed in such experiments require further validation.

Mammalian viruses and host RNAi

Several speakers provided intriguing new evidence that, akin to the situation in plants and insects, human viral pathogens also harness and manipulate the host RNAi machinery for their own agenda. Aniello Russo (Second U. Naples, Italy) identified miR-125a as a host-encoded regulator of hepatitis B virus (HBV). His data suggest a complex interaction between host and virus: whilst miR-125a is increased in HBV-infected cells and patients, it also binds to the viral RNA to suppress HBV gene expression. This self-inhibitory feedback loop might be key to chronic infection. A similarly intricate scenario was proposed by Kuan-Teh Jeang (NIH, USA), who identified a long non-coding (lnc) RNA in the host that is upregulated on HIV infection and that affects nuclear paraspeckles. Accordingly, he proposed that lncRNA–paraspeckle interactions might retain unspliced HIV RNA and thus contribute to latency. Similarly, Yamina Bennasser (Centre national de la recherche scientifique) discussed four cellular miRNAs that are upregulated in HIV-infected cells. Interestingly, these miRNAs activated rather than suppressed HIV replication. This was validated by delivering all four miRNAs to latently infected peripheral blood mononuclear cells from HIV-infected subjects, which reactivated HIV and forced it out of latency. Instead of the viral RNA, the miRNAs target nine cellular genes that might encode new antiviral proteins acting throughout the viral cycle. Equally exciting was a talk by Sébastien Pfeffer (U. Strasbourg, France) on the mutual interplay between mouse

cytomegalovirus and cellular miR-27. His data support a new mechanism of how viruses regulate host RNAi: namely, by encoding a transcript (m169) with a decoy miRNA binding site that triggers miRNA degradation [5]. The finding that miR-27 in turn controls the abundance of the m169 transcript raised the interesting question of whether it is more essential for the virus to regulate the miRNA, or whether it exploits a highly abundant cellular miRNA for its own regulation. This conundrum exemplifies perfectly the type of exhilarating hypotheses that were discussed at the meeting.

RNAi as antiviral tool in mammals

Next to these fundamental studies, many speakers focused on the exploitation and engineering of RNAi as an antiviral tool. Mark Kay (Stanford U., USA) screened a collection of short hairpin RNAs (shRNA) to dissect whether both RNA strands of hepatitis C virus (HCV) can be targeted by therapeutic RNAi; he tailored shRNAs to distinguish the two viral RNA strands. The key finding, that only the (+) strand can be targeted efficiently, led Kay to propose that the (–) strand might not be bioaccessible. An alternative means to achieve shRNA strand specificity was presented by Ying-Poi Liu from Berkhout's lab (U. Amsterdam, The Netherlands). Whilst testing a variety of shRNAs differing in stem–loop sequence and length, she noted that those with a 17–19 nt stem were processed by Ago2 rather than Dicer. By cleaving the 3′ arm, Ago2 released a defined, biologically active 30 nt product from these 'AgoshRNAs'. Their lab is further developing these promising new RNAi triggers as specific anti-HIV therapeutics. This might complement strategies presented separately by Berkhout to select potent anti-HIV shRNAs and accessible targets within the HIV genome using SHAPE technology [6]. Yet another approach to increase shRNA specificity was presented by Dirk Grimm (U. Heidelberg, Germany) who designed adeno-associated viral (AAV) vectors that co-express shRNAs with decoys that inactivate the shRNA passenger strand. Indeed, cDNA profiling of vector-treated cells confirmed that shRNA passenger strands suppress many unintended targets, and that this adverse event is counteracted effectively by the new decoys. Both Kay and Grimm moreover showed that RNAi specificity can be further enhanced by optimizing AAV vector tropism through molecular protein evolution.

Still, a remaining concern is mutational escape of the viral target. Several labs thus pursue combinatorial RNAi strategies that merge multiple RNAi triggers, or combine RNAi with other technologies. As one example, Kurreck targeted coxsackievirus B3 with siRNA or shRNA-expressing AAVs plus a soluble CAR receptor fused to an Fc domain expressed from an adenoviral vector. Puri Fortes (U. Navarro, Spain) instead combined shRNAs with anti-HBV inhibitors derived from nuclear U1 snRNPs. Curiously, RNAi and U1 only act synergistically if their targets do not overlap, implying that both mechanisms coexist in the cytoplasm, and raising interesting new questions on the function of U1 complexes.

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Another strategy to avert viral escape is to target cellular host factors instead of viral RNA. For instance, Alexander Karlas (MPI for Infection Biology, Berlin, Germany) used RNAi screening to unravel host restriction factors for influenza A virus. By targeting more than 23,000 genes with over 60,000 siRNAs, he identified and annotated 168 validated hits. As shown for one lead candidate, their inhibition blocked viral replication, proving the power of RNAi screens to discover antiviral targets. Alternatively, virus-associated miRNAs might provide promising targets for therapeutic intervention. Anna Kurzynska-Kokorniak (Institute of Bioorganic Chemistry, Poland) used SELEX to identify RNA oligomers that bind to Dicer. Some of them also selectively blocked the biogenesis of specific miRNAs, suggesting that her strategy is promising for the future targeting of virus-associated miRNAs. Along these lines, Troels Koch (Santaris Pharma, Denmark) described the company's latest clinical evaluation of an LNA inhibitor of miR-122—a hepatic miRNA and crucial host factor for HCV. Impressively, the mean HCV RNA reduction was 2.73 logs in the high-dose group, and HCV RNA became undetectable in 4 out of 9 patients in this cohort after one month of weekly subcutaneous LNA inhibitor administration. The LNA inhibitor was generally well-tolerated and no liver toxicity was detected. Future studies will indicate whether this new therapy can ultimately minimize the need for conventional interferon treatment of HCV patients.

Participants of the ESF–EMBO meeting in Poland spent their days discussing the latest results in the field of antiviral RNAi, and their nights rooting for their national football teams. Although the EURO 2012 football championship might not have been a success for everyone's favourite team, the exciting science and new concepts discussed at the conference certainly made all attendees return home feeling like winners.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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