Cell News

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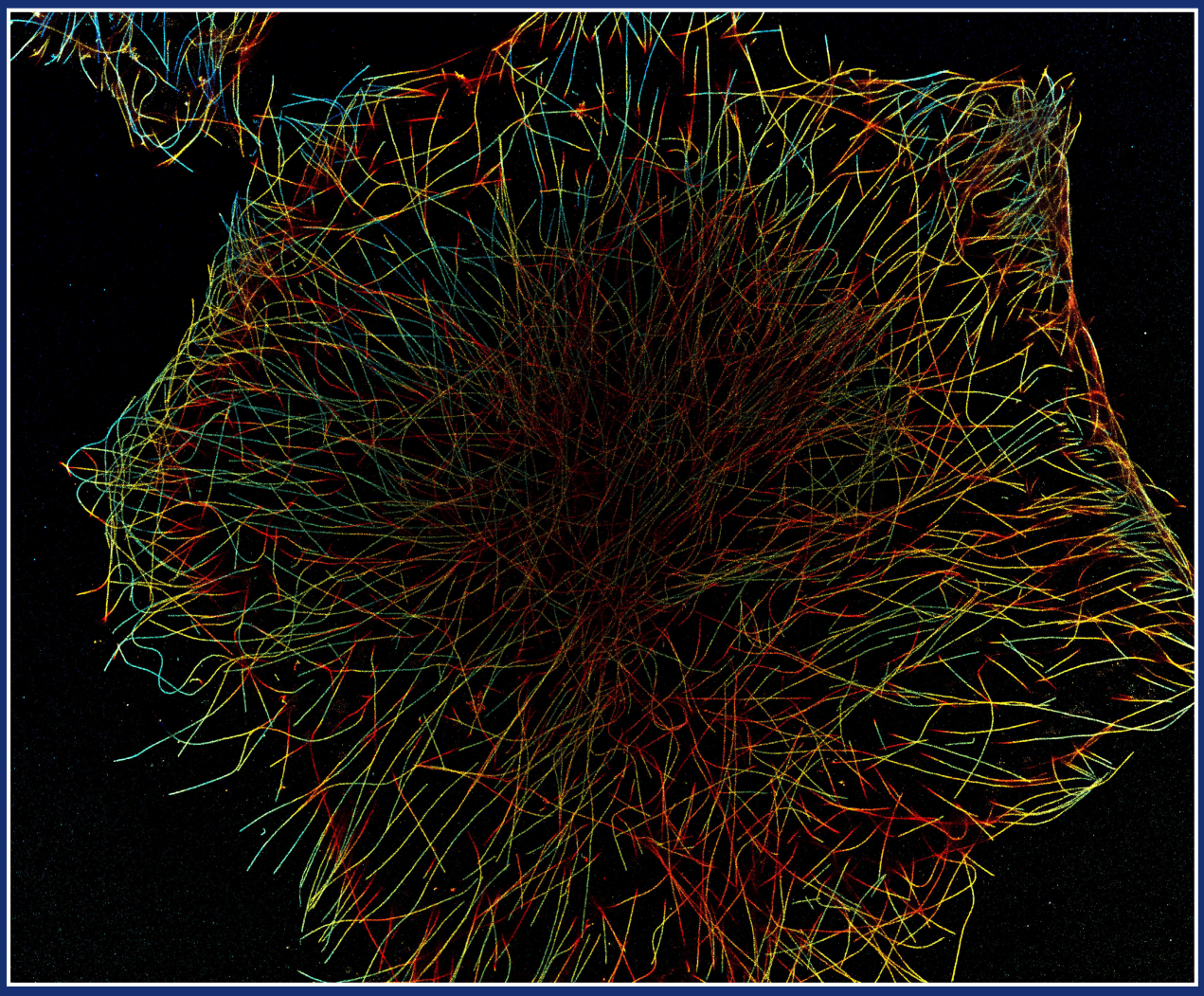

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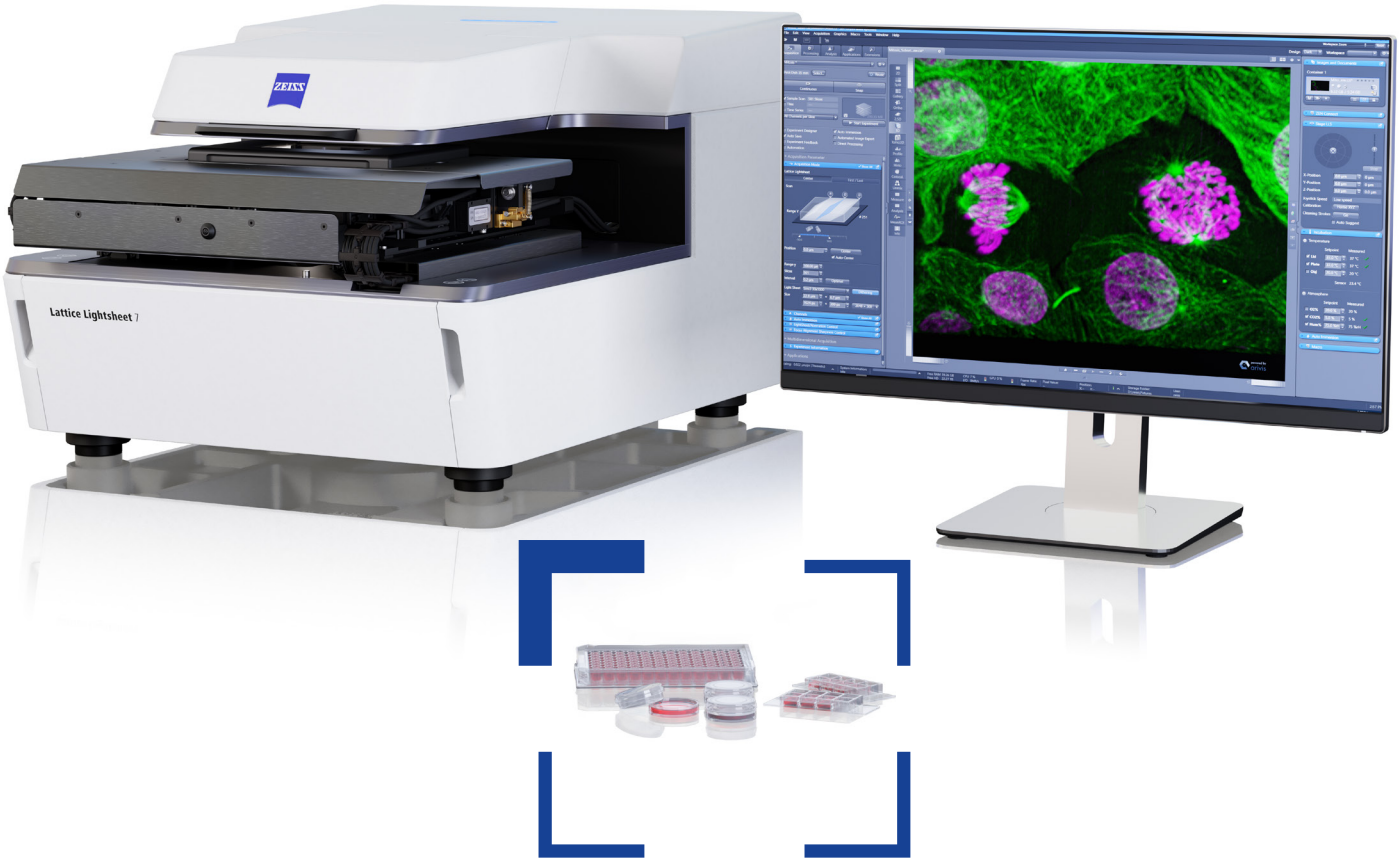
DGZ Prize Winners 2022

Discovering the subcellular dynamics of life.

DEUTSCHER ZUKUNFTSPREIS
Preis des Bundespräsidenten
für Technik und Innovation

Awardee 2022

The Federal President's Award
for Innovation and Technology



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Seeing beyond

CONTENT

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Cover Image:

3D DNA-PAINT super-resolution reconstruction of a cell's microtubuli network.
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Dear members and friends of the German Society for Cell Biology,

The year 2022 is coming to a close and for more than one reason we reflect on our community, how it began and how it is thriving today. First of all, there is sad news. Werner Franke, co-founder and a former president of the DGZ has recently passed away. Many of us didn't get the opportunity to meet him. But through a collection of eulogies by former colleagues and friends that we are currently assembling for our webpage you will still have a chance to get to know this remarkable man and scientist, to whom the whole field of cell biology owes so much. Our thoughts are with his family.

The end of the year also marks the end of the 2-year-term of the current DGZ board, Roland Wedlich-Söldner, Gislene Pereira, Sandra Iden, and Ralf Jungmann. When we started out, we took on the difficult task to increase the visibility of our society among the German scientific community and find ways to more actively engage our members. Despite the additional stress of the pandemic our efforts were very successful and the DGZ presents itself today as a growing and highly active community. This is only possible because many among you were extremely generous with their time and ideas – a big thank you to you all!

Let´s recap: We launched the **DGZ Focus Workshop series** (Zoom, last Tuesday each month, at 12 noon; https://zellbiologie.de/dgz-focus-workshops). Each workshop is organized by the two spokespersons of the corresponding **DGZ work group**. Invited speakers range from late-stage PhD students to established PIs with a focus on diversity regarding aspects like gender and host institution. The workshops have been a great success and an opportunity to learn about exciting research, to meet new people and to re-connect with friends and collaborators. We will continue the series in 2023 and strongly encourage everyone who hasn´t done so already, to participate and find out for themselves what it is all about. We aim to promote inclusion and attract new members, especially among young scientists, which is why the series is open to everyone: please spread the news!

Scientific awards 2022: On November 22nd, we celebrated outstanding scientific achievements – across the whole range of career stages – in our annual DGZ prize webinar. The Nikon Young Scientist Award went to Jan Hansen for his PhD work in the lab of Dagmar Wachten at the University of Bonn. The Werner Risau Prize was awarded to Andrew Chris Yang from the University of California, San Francisco. Andrew Clark from the Universities of Stuttgart and Tübingen received the Walther Flemming Award and Lena Pernas from the MPI for Biology of Aging in Cologne is the recipient of the BINDER Innovation Prize. Finally, Ewa Paluch from the University of Cambridge presented her research on the role of physical forces during cellular morphogenesis in the Carl Zeiss Lecture 2022. We want to thank Carl Zeiss, Nikon, BINDER, ibidi and the Werner Risau committee for their continued and generous sponsorship.

Unfortunately, not everything went as smoothly. The long-awaited DGZ website should have been up and running by now and reflect the new structure with our 12 working groups. Due to some technical difficulties, the project was delayed but should now be – fingers crossed! - launched in time for our members meeting on December 15th. For more information on workshops and all other activities, you can also follow us @DGZ_info on twitter.

What to expect in 2023?

Preparations for the **International DGZ Meeting in Saarbrücken from October 10th–13th 2023** are well advanced. Under the overarching theme of "Cell Physics" the DGZ will join forces with the SFB1027 – "Physical modeling of non-equilibrium processes in biological systems". After long abstinence we are very much looking forward to an in-person meeting again. For any questions regarding the meeting, please contact Sandra Iden.

As always please feel free to share your thoughts and your criticisms on our activities.

Stay tuned and join us in shaping the future of cell biology in Germany.

The DGZ board!

IN MEMORIAM

Prof. Dr. Werner W. Franke

1940 – 2022

The DGZ mourns the sudden death of Prof. Werner Franke. He was not only a renowned cell biologist and co-founder of the DGZ but also acted as a pioneering and very passionate anti-doping activist.
Our feelings are with his family and friends.

DGZ Mitgliederversammlung 2022

Liebe Mitglieder der Deutschen Gesellschaft für Zellbiologie!

Wir laden Sie ein zur diesjährigen Mitgliederversammlung der DGZ, die am

15. Dezember 2022, 12.00 Uhr – 14.00 Uhr
online über zoom

stattfinden wird. Die Meeting ID und das Passwort werden allen Mitgliedern rechtzeitig per E-Mail zugeschickt.

Tagesordnung:

1. Bestätigung des Protokolls der letzten Sitzung
2. Jahresbericht des Präsidenten mit anschließender Diskussion
3. Geschäfts- und Kassenbericht über das abgelaufene Kalenderjahr
4. Bericht der Rechnungsprüfer:in
5. Entlastung des Vorstandes
6. DGZ Wahlen 2022-2024
7. Festsetzung der jährlichen Mitgliedsbeiträge
8. Sonstiges

DGZ Member Meeting 2022

Dear Members of the German Society for Cell Biology!

We would like to invite you to this years' member meeting of the DGZ, which is going to take place on

December 15, 2022, 12:00 h – 14:00 h
online via zoom

The meeting ID and password will be communicated via individual e-mail to all members in due time.

Agenda:

1. Confirmation of the minutes of the last meeting
2. Annual report of the president with discussion
3. Report on finances of the past calendar year
4. Auditors' report
5. Discharge of the board
6. DGZ election 2022-2024
7. Designation of annual membership fees
8. Other items

WALTHER FLEMMING AWARD

Mechanical feedback during collective cell migration

Andrew G. Clark

Introduction

Collective cell migration is an essential biological process involved in development, tissue repair and cancer metastasis[1]. In early stages of metastasis in solid tumors, cancer cells break away from the primary tumor and migrate through the surrounding stromal tissue, which comprises primarily collagen-I extracellular matrix networks and is home to a number of different cell types that make up the tumor microenvironment. It is becoming increasingly clear that this process involves not only single cell migration, but is often associated with collective migration of groups of cells. Recent work has suggested that such collective tumor invasion is correlated with poor prognosis and that groups of cells have enhanced metastatic potential compared to cancer cells migrating individually[2,3]. However, the mechanisms underlying the difference between single-cell and collective invasion are poorly understood.

The mechanical and topological properties of stroma-like collagen networks can tune cell migration dynamics[4]. For example, cells embedded in networks of aligned collagen fibers preferentially migrate along the axis of fiber alignment[5]. Such alignment of collagen networks also occurs during cancer progression, and the presence of aligned collagen bundles is associated with poor prognosis[6]. In addition to cells responding to physical cues from collagen networks, cells themselves can exert sufficient mechanical forces to modify the local network structure. Large groups of cells in or top of collagen networks generate pulling forces that result in radially aligned collagen bundles, which then facilitate invasion of single cells into the surrounding matrix after the initial alignment phase[7,8]. However, the mechanical feedback between cells and collagen networks during collective migration is not well understood. In other words: how do groups of cells physically remodel local collagen networks, and how does the modified collagen structure in turn affect migration dynamics?

Cell clusters generate collagen gradients during collective migration

To study collective migration on collagen networks, we generated small clusters of A431 cells and seeded them on top of in vitro polymerized collagen networks (Fig. 1a). Cell clusters attached to the underlying collagen networks and retained an intact cluster structure (Fig. 1b). To establish how collagen network structure affected collective migration dynamics, we seeded the cell clusters on top of soft poly-A-acrylamide (PAA) gels coated with either a thin collagen network or monomeric collagen (Fig. 1c). Using live imaging and cell tracking analysis, we found that cell clusters on the collagen networks migrated with

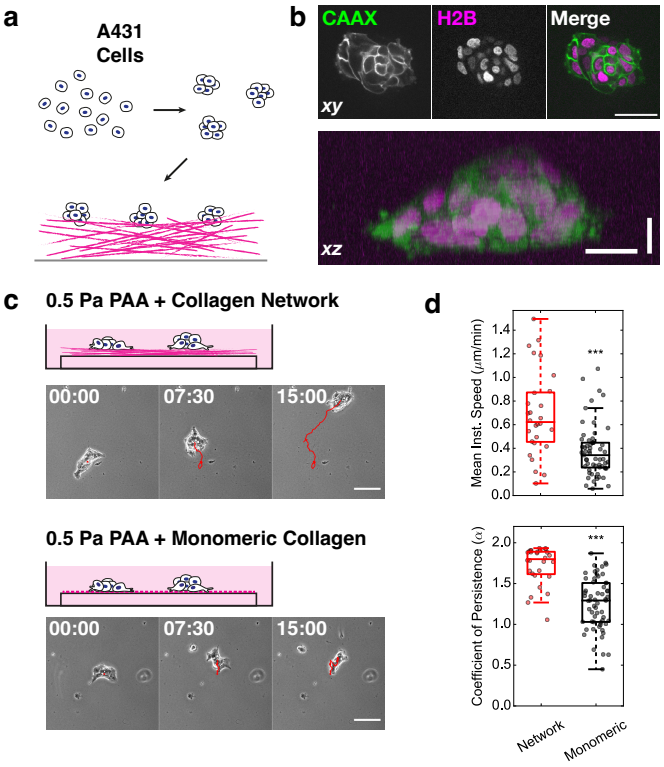

Figure 1. Cell clusters migrate persistently on collagen networks. (a) Schematic describing formation and seeding of cell clusters on collagen-I networks. **(b)** Micrograph of A431 clusters expressing GFP-CAAX (Plasma Membrane) and mCherry-H2B (DNA). Horizontal scale bars: 50 µm, 20 µm. Vertical scale bar: 100 µm. **(c)** Schematics and montages of A431 clusters migrating on 0.5 kPa PAA gels coated with a thin 2 mg/ml collagen-I network (top panels) or 100 µg/ml monomeric collagen-I (bottom panels). Scale bars: 100 µm. Time in hours:minutes. **(d)** Box plots of migration speed and persistence for clusters on PAA gels coated with thin collagen networks or monomeric collagen. ***p<0.001 for Welch's t-test. Adapted from[17].

higher speed and persistence, meaning that they migrated along straighter trajectories (Fig. 1d). These data suggested that the structure and mechanical properties of the collagen networks promoted more persistent collective migration.

We next investigated how cells locally reorganized collagen networks during migration. To this end, we performed live imaging of cell clusters migrating on fluorescently labeled collagen networks (Fig. 2a). By analyzing the collagen structure under the cluster, we found that collagen density reached a maximum that was offset toward the trailing edge (rear) of the cluster, while collagen fiber alignment reached a minimum that was also offset toward the cluster rear (Fig. 2b,c). To better understand how these gradients in collagen structure were related to mechanics,

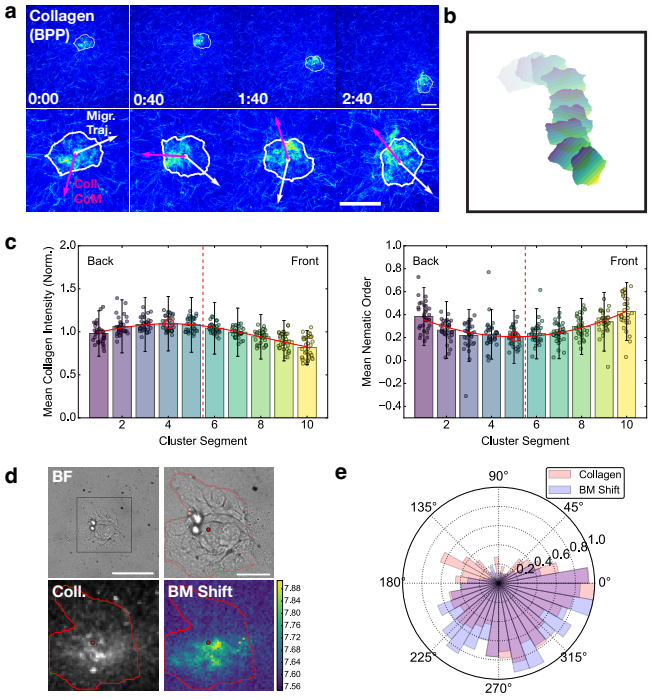

Figure 2. Cell clusters generate collagen gradients during collective migration. (a) Pseudocolour montage of a cell cluster migrating on a fluorescent collagen network (brightest point projection, BPP). White lines: cluster contour. White dots: cluster center of mass (CoM). White arrows: migration trajectory. Magenta arrows: direction of collagen density center of mass. *Lower panel*: zoom from upper panel. Scale bars: 25 µm. **(b)** Segmentations of the cell cluster from the time series in a, binned from the cluster front (yellow) to the rear (violet). **(c)** Bar plots of collagen intensity and alignment (nematic order) over cluster regions (mean ± s.d.). Red lines: Gaussian and inverted Gaussian fits to the mean values. Red circles: mean position, µ, from fits. **(d)** Micrographs showing collagen density and BM shift (a proxy for stiffness). Red lines: cluster contour. Red dots: cluster center of mass. Scale bars: 50 µm, 20 µm. Colour bar: Brillouin shift (GHz). **(e)** Polar histogram of maximum collagen density (red) and BM shift (blue) at different angles from the center of mass of the cluster in d. Data were rescaled to the range 0–1 to exemplify regional differences. Adapted from[17].

we analyzed collagen networks using Brillouin microscopy, a non-invasive technique to measure viscoelastic material properties[9]. We found that regions of high collagen density correlated with higher Brillouin shift, a proxy for mechanical stiffness (Fig. 2d,e). Together, these data suggested that cell clusters generate gradients in collagen density, alignment and stiffness during collective migration. While cell clusters migrated down gradients in collagen density and stiffness, they migrated up a gradient in collagen fiber alignment.

Collective migration dynamics depend on collagen network viscoelasticity

We next sought to understand how the mechanical properties of collagen networks could influence the formation of collagen gradients, and how these gradients in turn could affect migration dynamics. To this end, we developed a simple theoretical model of collective migration that included mechanical feedback between the cell cluster and its underlying substrate (Fig. 3a). The model suggested that migration persistence was dependent on substrate viscoelasticity (Fig. 3b). Our model predicted that while clusters on a viscoelastic substrate could migrate in a spontaneously persistent manner, clusters migrating on a purely elastic substrate would not migrate persistently (Fig. 3c). This notion was consistent our initial measurements of collective migration dynamics on viscoelastic collagen networks vs. elastic PAA gels (Fig. 1c,d). In addition, the model suggested that migration on a viscoelastic substrate would result in a peak in substrate deformation that was offset toward the cluster rear (Fig. 3c), consistent with our analysis of fluorescent collagen networks during cluster migration (Fig. 2a-c).

Previous rheological studies have suggested that collagen networks behave in a highly non-linear manner and display viscoelastic behavior that depends on both the magnitude and timescale of mechanical stress exerted on the collagen network[10–13]. To investigate the rheological properties of collagen networks in response to mechanical forces generated by cell clusters, we developed an assay to measure collagen relaxation following the rapid loss of cell-generated forces. We seeded cell clusters on PAA gels coated with thin fluorescent collagen networks and allowed the clusters to mechanically reorganize the collagen. We then quickly removed the cells by adding Trypsin and Ammonium Hydroxide (NH4OH; Fig. 3d). The deformations in the PAA gel relaxed nearly instantaneously, indicating a rapid loss of mechanical stresses exerted by the cells. Measuring the decrease in collagen density and collagen movements by particle image velocimetry (PIV), we found that the collagen network relaxed much more gradually due to its viscoelastic nature (Fig. 3e). By fitting the collagen density relaxation curves, we could extract a viscoelastic relaxation time of ~10min for the collagen networks.

The theoretical model predicted that reducing the viscoelastic relaxation time of the substrate would result in more symmetric

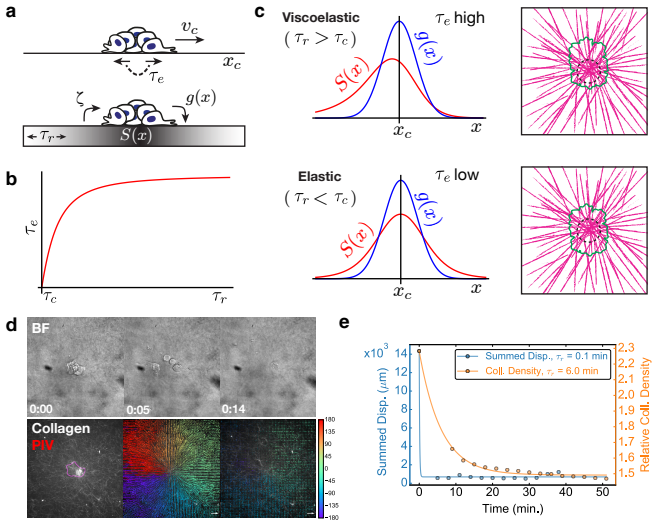

Figure 3. Collagen viscoelasticity enhances migration persistence. (a) Schematic description of a theoretical model of cell cluster migration on a viscoelastic substrate. Importantly, the model includes active deformation of the substrate by the cell cluster and feedback from the substrate deformation on cluster migration. **(d)** Plot of migration persistence (given by the timescale of directional switching, τ_e) vs. the substrate viscoelastic timescale, τ_r. (c) Interpretation and predictions of the model. For clusters migrating on viscoelastic collagen networks with sufficient relaxation time ($\tau_r > \tau_c$), clusters are expected to migrate persistently (with high τ_e) due to the generation of a collagen gradient. For more elastic substrates ($\tau_r < \tau_c$) collagen deformation remains symmetric, and low persistence migration (low τ_e) is predicted. (d) Montage of brightfield and fluorescent collagen networks with rapid cell removal by trypsin/NH_4OH treatment. Arrows: collagen movements analyzed by PIV. Magenta line: cell contour. Scale bar: 50 μm. Scale vector: 0.5 μm/min. Time in hours:minutes after addition of trypsin/NH_4OH. Time 0:00 indicates just prior to addition of trypsin/NH_4OH. **(e)** Plot of summed 3D displacements of the PAA gel (a proxy for mechanical force production) and collagen density within the cell contour region following trypsin/NH_4OH treatment. Dots: density at individual time points. Lines: exponential decay fit to extract relaxation time, τ_r. Adapted from[17].

collagen density profiles and lower migration persistence. To test this prediction experimentally, we crosslinked fluorescent collagen networks by treating with the small sugar Threose or with Glutaraldehyde to reduce the viscoelastic relaxation time. Using the rapid cell removal assay, we confirmed that crosslinking indeed reduced collagen network relaxation time (Fig. 4a). Consistent with our theoretical model, we found that cell clusters on crosslinked networks generated shallower, more symmetric collagen gradients and migrated less persistently (Fig. 4b-d).

Collective migration is more efficient than single cell migration

Our theoretical model made an additional important prediction, namely that small clusters and single cells would migrate less persistently compared to large clusters. This prediction is based on two general assumptions: (1) larger clusters generate more mechanical force and reorganize collagen networks to a larger degree and (2) single cells and small clusters are too small to sense collagen gradients. We next experimentally tested these assumptions. To determine how mechanical force scales with cluster size, we seeded single cells and cell clusters on PAA gels coated with thin collagen networks and measured the displacement of the PAA gels to infer mechanical stress. We found that substrate displacement scaled with cluster size, with larger clusters producing larger displacements, consistent with previous studies[14] (Fig. 5a). We next seeded single cells on fluorescent collagen networks and measured average collagen density along the length of the cell. While cell clusters formed collagen gradients during migration, the collagen density profile along the length of single cells was flat, indicating that single cells were indeed too small to sense collagen gradients (Fig. 5b). We next compared migration dynamics of single cells vs. clusters by live imaging and cell tracking. We found that while cell clusters migrated in a persistent manner, single cells migrated in an erratic, non-persistent fashion, consistent with the prediction of our theoretical model (Fig. 5c).

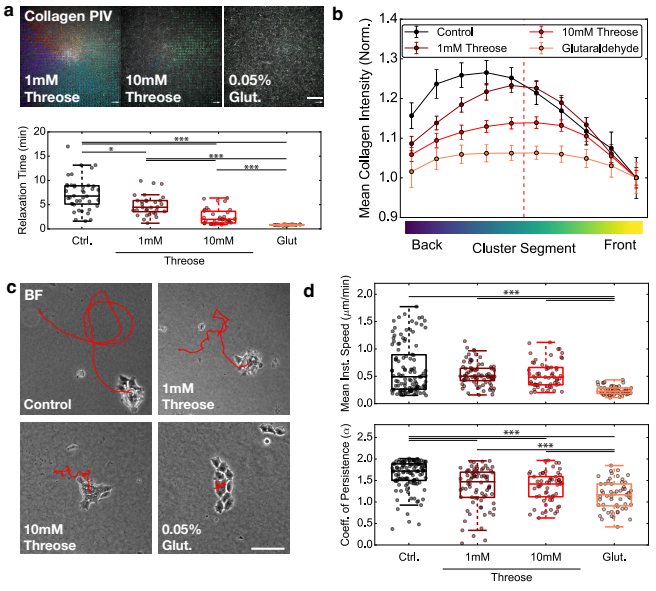

Figure 4. Collagen crosslinking decreases viscoelastic relaxation time and reduces migration persistence. (a) PIV analysis of fluorescent collagen movements ~12 min after cell removal by trypsin/NH_4OH treatment. Scale bar: 50 μm. Scale vectors: 0.5 μm/min. *Lower panel*: box plot of collagen relaxation time, τ_r, with collagen crosslinking. **(b)** Plot of collagen intensity along the cluster length for clusters migrating on crosslinked fluorescent collagen networks (from live imaging of cluster migration on fluorescent collagen networks). **(c)** Brightfield micrographs from cell cluster migration on crosslinked collagen networks after 16 h of migration. Red lines: cluster trajectories. Scale bar: 100 μm. **(d)** Box plots of mean instantaneous speed and coefficient of persistence for clusters migrating on crosslinked collagen networks. *p<0.05,***p<0.001 for Tukey HSD test. Adapted from[17].

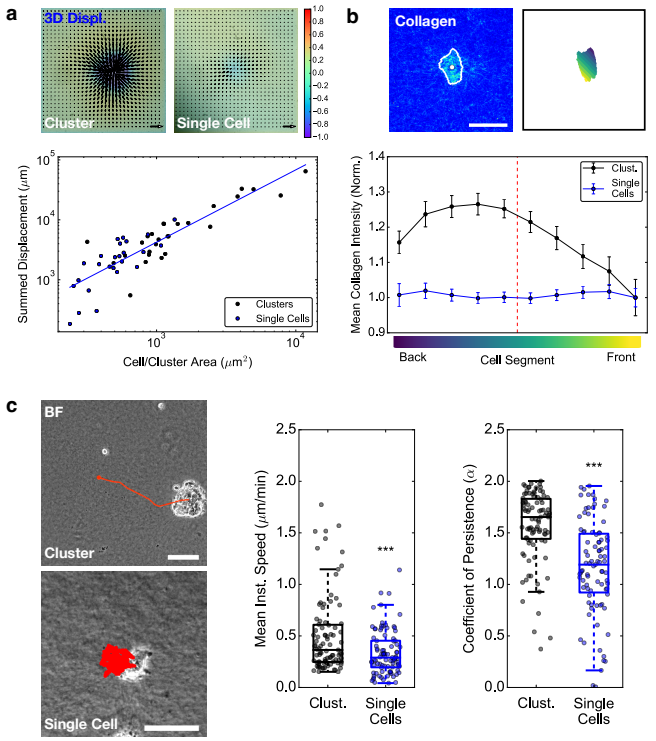

Figure 5. Single cells migrate less persistently than cell clusters. (a) 3D displacement microscopy comparing clusters and single cells. Black arrows: *xy* displacements. Color scale: z displacements (µm). Scale vector: 5 µm. *Lower panel*, Plot of 3D displacements vs. cell or cluster area. Blue line: power-law fit (all data). (b) *Left*: Micrograph of a single cell migrating on a fluorescent collagen network. White line: cell contour. White dot: center of mass. Scale bar: 50 µm. *Right*: cell segmentation binned over cluster length. *Lower panel*: plot of collagen intensity along cell/cluster length (mean ± s.e.m.). (c) Brightfield images of a cluster and single cell migrating on collagen networks. Red line: cell trajectory. *Right*: Box plots of migration speed and persistence for clusters vs. single cells. ***p<0.001 for Welch's t-test. Adapted from[17].

Conclusion

In this study, we provide a simple physical mechanism for persistent migration of cell clusters on collagen networks, whereby cell clusters generate gradients in collagen density, alignment and stiffness during migration; these gradients, in turn, promote persistent collective migration. This model relies not on biochemical polarity or signaling mechanisms, but rather on the mechanical feedback between groups of cells and their substrate. This mechanism is analogous to other recent work describing self-generated gradients by cells migrating in uniform concentrations of chemokines and by non-living isotropic particles that spontaneously migrate in solution[15,16]. This suggests that such simple physical models can have wide-reaching applications.

Our model also provides a mechanism to explain why cell clusters migrate more efficiently than single cells, which could contribute to the increased metastatic potential of cancer cell clusters. Because this model relies simply on the physical interactions between cells and their substrate, it can be applied to a number of different cancer types despite differences in their mutation and signaling profiles. In addition, our work shows that simply by tuning collagen network mechanics, we can dramatically influence cell migration patterns. Changes in tissue mechanics is associated with a number of pathologies including chronic inflammation and cancer, although the effects of these mechanical changes are not well understood. Questions related to how cells physically interact with and respond to their microenvironment continues to be an area of intense research and has medically relevant implications for a number of diseases. Future studies using both basic and translational research approaches will help to understand how we can harness cell and tissue mechanics as both a prognostic marker and a potential treatment strategy.

Acknowledgements

I would like to acknowledge all co-authors and collaborators that contributed to this work and made this collaborative project possible[17]. In particular, I thank my postdoctoral supervisor Danijela Matic Vignjevic for allowing me the freedom to explore different projects and collaborations during my postdoc and for always providing the support and advice I needed along the way. I would like to also thank previous supervisors, mentors and collaborators for their belief and support. A big thanks to Bill Bement, Ann Miller, Ewa Paluch, Guillaume Salbreux and Kai Dierkes for guiding me and teaching me everything I know. I would like to acknowledge everyone at the University of Stuttgart and University of Tübingen for their continual support and for the opportunity to start a lab of my own. I especially thank the first-generation Clark lab members Thao Nguyen, Sarbari Saha and Fabian Gärtner for their bravery in joining such a young lab and for their perseverance and positive attitudes that have allowed us to kick-start exciting new projects in the lab. I thank the DGZ and ibidi for honoring me with this award as well as funding bodies that have supported my past and current research, including the HFSP, ERC, EMBO, BMBF and MWK. Finally, I would like to thank my family, Carmen, Luis and Maxine for their constant love, support and encouragement along the way.

References

1. Friedl, P. & Gilmour, D. Collective cell migration in morphogenesis, regeneration and cancer. *Nat Rev Mol Cell Biol* **10**, 445–457 (2009).
2. Ohike, N. *et al.* Tumor Budding as a Strong Prognostic Indicator in Invasive Ampullary Adenocarcinomas. *Am J Surg Pathol* **34**, (2010).
3. Aceto, N. *et al.* Circulating Tumor Cell Clusters Are Oligoclonal Precursors of Breast Cancer Metastasis. *Cell* **158**, 1110–1122 (2014).
4. Clark, A. G. & Vignjevic, D. M. Modes of cancer cell invasion and the role of the microenvironment. *Curr Opin Cell Biol* **36**, 13–22 (2015).
5. Riching, K. M. *et al.* 3D Collagen Alignment Limits Protrusions to Enhance Breast Cancer Cell Persistence. *Biophys J* **107**, 2546–2558 (2014).
6. Conklin, M. W. *et al.* Aligned Collagen Is a Prognostic Signature for Survival in Human Breast Carcinoma. *Am J Pathol* **178**, 1221–1232 (2011).
7. Shi, Q. *et al.* Rapid disorganization of mechanically interacting systems of mammary acini. *Proc. Natl. Acad. Sci.* USA **111**, 658–663 (2014).
8. Kopanska, K. S., Alcheikh, Y., Staneva, R., Vignjevic, D. & Betz, T. Tensile Forces Originating from Cancer Spheroids Facilitate Tumor Invasion. *PLoS One* **11**, e0156442 (2016).

9. Prevedel, R., Diz-Muñoz, A., Ruocco, G. & Antonacci, G. Brillouin microscopy: an emerging tool for mechanobiology. *Nat Methods* **16**, 969–977 (2019).
10. Pryse, K. M., Nekouzadeh, A., Genin, G. M., Elson, E. L. & Zahalak, G. I. Incremental Mechanics of Collagen Gels: New Experiments and a New Viscoelastic Model. *Ann Biomed Eng* **31**, 1287–1296 (2003).
11. Xu, B., Li, H. & Zhang, Y. Understanding the viscoelastic behavior of collagen matrices through relaxation time distribution spectrum. *Biomatter* **3**, e24651 (2013).
12. Licup, A. J. *et al.* Stress controls the mechanics of collagen networks. *Proc. Natl. Acad. Sci. USA* **112**, 9573–9578 (2015).
13. Pajic-Lijakovic, I., Milivojevic, M. & Clark, A. G. Collective Cell Migration on Collagen-I Networks: The Impact of Matrix Viscoelasticity. *Front Cell Dev Biol* **10**, 901026 (2022).
14. Mertz, A. F. *et al.* Scaling of Traction Forces with the Size of Cohesive Cell Colonies. *Phys Rev Lett* **108**, 198101 (2012).
15. Michelin, S., Lauga, E. & Bartolo, D. Spontaneous autophoretic motion of isotropic particles. *Phys. Fluids* **25**, 061701 (2013).
16. Tweedy, L. *et al.* Seeing around corners: Cells solve mazes and respond at a distance using attractant breakdown. *Science (1979)* **369**, eaay9792 (2020).
17. Clark, A. G. *et al.* Self-generated gradients steer collective migration on viscoelastic collagen networks. *Nat Mater* **21**, 1200–1210 (2022).

CV/Biosketch

Andrew received his Bachelor's degree in Molecular Biology from the University of Wisconsin-Madison and studied epithelial wound repair in the lab of Bill Bement. He then moved to the Max Planck Institute for Molecular Cell Biology and Genetics in Dresden, where he did his PhD studying the organization and mechanics of the cellular actin cortex in the lab of Ewa Paluch. Following a short follow-up postdoc at University College London, he moved to Paris to do a postdoc at the Institut Curie in the lab of Danijela Matic Vignjevic. During his postdoc, Andrew studied collective cell migration in a number of contexts, including using microfluidic devices to study collective chemotaxis, mechanical interactions between cells and ECM networks during migration and collective migration in the intestinal epithelium. Andrew started his own group in May 2021 as a joint position between the University of Stuttgart, Institute for Cell Biology and Immunology and the University of Tübingen, Center for Personalized Medicine, where his lab studies intestinal epithelial dynamics in physiology and disease.

https://www.izi.uni-stuttgart.de/en/research/clark/
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NIKON YOUNG SCIENTIST AWARD

Ciliary cAMP signaling – cellular functions and mechanisms

Jan N. Hansen

How do cells perceive their environment and react to it? In this context, one organelle particular stands out: the cilium. Cilia are tiny, hair-like cellular appendages that emanate from the surface of most vertebrate cells. Cilia are classified into motile cilia and immotile, so-called primary cilia. Motile cilia actively move. Their characteristic beating generates fluid flow on epithelia or propels entire cells, such as sperm cells. Primary cilia appear solitary, on a variety of cells through our body, such as kidney epithelial cells or neurons. Cilia not only look like cellular antenna – they are cellular antenna. Both, motile and primary cilia, sense extracellular stimuli and transduce this information separately from the cell body into a cellular response (Anvarian et al., 2019; Nachury and Mick, 2019; Wachten et al., 2017) (Fig. 1). However, which cellular functions are controlled by ciliary signaling as well as the molecular mechanisms underlying the signal transduction remain enigmatic – in particular, for primary cilia.

Cyclic AMP (cAMP) has been proposed as a central component of signaling in both, motile and primary cilia. Whereas the molecular players and cellular outputs have mostly been described for motile cilia, i.e., for sperm flagella (Wachten et al., 2017), this information is lacking by large for primary cilia. In my PhD project, I aimed to shed light on cAMP signaling in motile and primary cilia.

Challenges: Flagella beat fast, primary cilia are tiny

The study of cilia and flagella faces several challenges. Sperm cells rapidly swim, and their flagella beat in three dimensions and extremely fast. Within a second, the flagellum of a human sperm cell beats ten to thirty times (Gallagher et al., 2019). Thus, we need high-speed 3D imaging and suitable image analysis methods to track the flagellar beating. In addition, tools are required that allow to analyze ciliary signaling separately from the cell body – in particular, for primary cilia. They comprise a volume that is 5,000-fold smaller than the cell body. Conventional pharmacological or genetic approaches affect the whole cell and thus, are not applicable.

I overcame these challenges by (1) developing new imaging and image analysis methods to characterize signaling and function of motile and primary cilia and (2) establishing an optogenetic approach to specifically manipulate ciliary cAMP signaling.

cAMP signaling is sub-compartmentalized in mammalian sperm flagella

The Wachten group initially established an optogenetic approach and biosensors to manipulate and measure ciliary cAMP levels in sperm flagella (Jansen et al., 2015; Mukherjee et al., 2016; Raju et al., 2019). Sperm use their flagellum as a sensor, computer, motor, and rudder – the flagellum contains a unique signaling architecture to transduce environmental cues into a

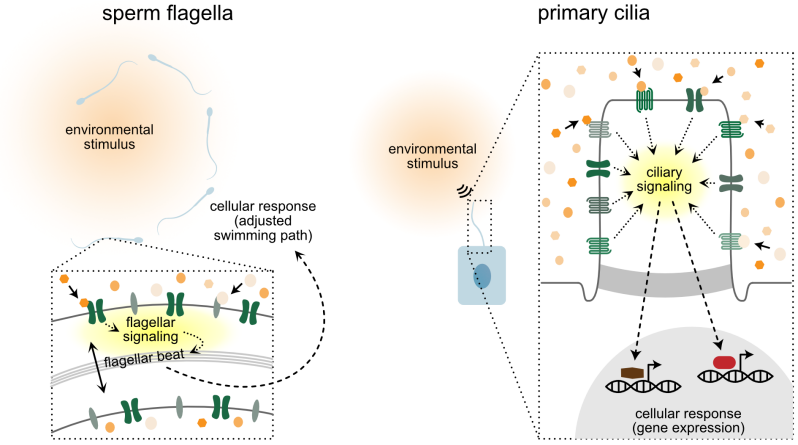

Figure 1: Cilia and flagella sense the environment, transduce the signals via a local signaling machinery, and trigger a cellular response, such as, changing gene expression or adjusting the swimming path. Figure derived from: Jan N. Hansen, Ciliary cAMP signaling - cellular function and mechanisms (2021, University of Bonn, PhD thesis).

specific flagellar beat and thereby, swimming response (Fig. 1) (Alvarez et al., 2014). cAMP signaling seems to play a role in this process in mammalian sperm (Balbach et al., 2018). However, spatial insight into how cAMP signals adjust the flagellar beat was missing due to the lack of suitable imaging and image analysis tools. To precisely characterize the flagellar beat in our optogenetic mouse models, I developed SpermQ (Hansen et al., 2019), an open-source analysis software to comprehensively characterize the beat of motile cilia from two-dimensional high-speed recordings, obtained with fluorescence, dark-, or bright-field microscopy. SpermQ cannot only be used for sperm flagella but also for other motile cilia – we also successfully applied SpermQ to reconstruct the beat of motile cilia in the brain of zebrafish larvae (Olstad et al., 2019).

Combining optogenetics and SpermQ allows to precisely characterize how adjustments of flagellar cAMP levels affect the flagellar beat. SpermQ allowed us to reveal that a cAMP stimulus induced via optogenetics resulted in a different modulation of the flagellar beat than a cAMP stimulus induced via bicarbonate (Raju et al., 2019), which activates the endogenous adenylyl cyclase ADCY10 in mammalian sperm flagella (Balbach et al., 2018). This highlights that the signaling machinery in the flagellum must be sub-compartmentalized into fine-tuned microdomains that lose control when we adjust cAMP along the flagellum using optogenetics. Our optogenetic approaches and SpermQ allow to dissect the unique microdomain architecture in more detail, with spatial resolution.

Relating flagellar signaling, flagellar beating, and swimming path

I next aimed to understand how specific flagellar beat patterns relate to the swimming path. Yet, all available techniques, including SpermQ, could never capture both, beat pattern and swimming path, for the same sperm cell and with high precision. In clinics and research, Computer Assisted Semen Analysis (CASA) machines are widely established albeit they mainly characterize the swimming path and do not fully capture flagellar beating (Baskaran et al., 2021). By contrast, techniques to quantify flagellar beating require high-resolution, high-speed imaging and thus, lack a sufficiently large field of view to observe free swimming sperm over many beat cycles. Thus, for quantifying the flagellar beat, it is common practice to record sperm that are tethered to the cover glass at the head. SpermQ allows to comprehensively characterize the flagellar beat from such data, but additional simulations and 3D tracking of the swimming trajectory need to be performed to relate the flagellar beating characteristics to the swimming path, as we demonstrated in a study on how tubulin glycylation fine-tunes the flagellar beat in mouse sperm (Gadadhar et al., 2021).

I aimed to develop an imaging technique that allows to directly relate flagellar beat and swimming path, for free swimming sperm. To capture the full detail of the beat, 3D imaging is essential since sperm of some species, i.e., human and mouse sperm, roll around their longitudinal axis while swimming (Miki and Clapham, 2013; Wachten et al., 2017) and thus, the reference system of the flagellar beat also rotates in 3D. Few imaging setups to digitalize the 3D nature of the flagellar beat had been built previously (Bukatin et al., 2015; Dardikman-Yoffe et al., 2020; Gong et al., 2021; Muschol et al., 2018), but they were limited by being biased by assumptions on the 3D shape of the flagellar beat (Bukatin et al., 2015), by a low temporal resolution (Muschol et al., 2018), or by a small field of view (Dardikman-Yoffe et al., 2020; Gong et al., 2021). Furthermore, these methods require computationally challenging image analysis, exhausting the capacities of standard labs. In contrast, I developed an imaging method that provides a large field of view (240x260 µm), high speed (500 frames per second), and good precision (down to 0.4 µm), being realized by simple insertion of a commercially available multifocal imaging device into a common brightfield microscope (Hansen et al., 2021a). This device allows to acquire four focal planes simultaneously. To achieve high precision tracking of the whole flagellum in z (= along the imaging axis) from only four focal planes, I developed an algorithm based on the principle of depth-from-defocus (Fig. 2A): the algorithm measures the width of the flagellar image in each focal plane and concludes the flagellar z position from the width, based on a previously determined calibrated relationship of flagellar width, position on the flagellum, and z position (Fig. 2B) (Hansen et al., 2021a). We observed that using multiple focal planes to infer the z position resulted in a better z precision and provided a wider depth with good z precision compared to using only one focal plane. We reached a localization precision in z of >= 0.4 across a range of about 20 µm with our four-plane imaging setup. This allowed us to characterize the flagellar beat of free swimming, human sperm over a time period of 2.5 seconds (Fig. 2C), allowing to visualize changes in the swimming pattern over time (Hansen et al., 2021a). Notably, we not only explored this technique for sperm flagella but also for high-speed 3D particle imaging velocimetry, whereby we experimentally generated the first high-resolution 3D fluid flow profile around a human sperm flagellum (Hansen et al., 2021a) and confirmed previous theoretical predictions (Ishimoto et al., 2017).

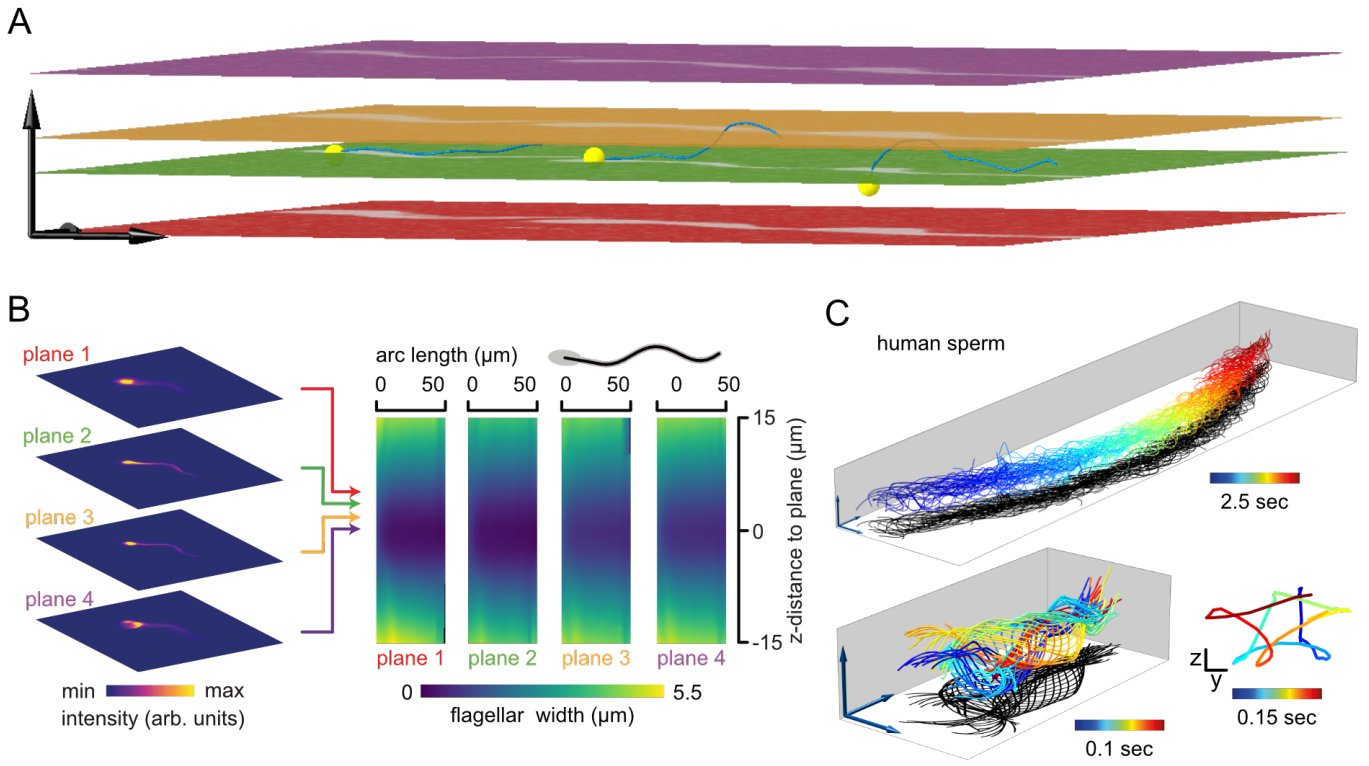

Figure 2: Multifocal imaging to digitalize the 3D flagellar beat of human sperm. (A) 3D visualization of the four planes (depicted in different colors) acquired by the multifocal imaging setup and of the flagellum reconstructed. Three exemplary timepoints are overlayed. Flagella are indicated in blue, positions of sperm heads are indicated as yellow spheres. Arrows indicate 20 µm. **(B)** Characterizing the relationship between the image and the z-position of human sperm flagella in the multifocal imaging setup. The flagellar width (color-coded) was determined by a Gaussian curve fit on a normal to the flagellum at different flagellar positions (arc length) and for different distances of the sperm flagellum to the respective plane (mean image of n = 12 immotile sperm from five different donors). When the flagellum is more remote from the focal plane it appears wider. The determined relationship tables of flagellar width, arc length, and z-distance to the plane are used by our algorithm to predict the z position based on the flagellar image in the four acquired planes. **(C)** 3D-tracking of a free swimming, human sperm. Views for visualizing the trajectory (top, only every 4th frame plotted for better visualization), the flagellar beat (bottom left), and sperm rolling (bottom right, yz-projection of a flagellar point at arc length 20 µm, view in swimming direction). Bars and arrows indicate 10 µm. Figures modified from Hansen et al., Nat. Commun., 2021, available at https://dx.doi.org/10.1038/s41467-021-24768-4, published under CC-BY 4.0 license (http://creativecommons.org/licenses/by/4.0/).

In summary, I established a method that allows a longitudinal analysis of flagellar signaling, flagellar beat, and swimming path, solving a long-standing challenge in sperm research. The newly developed tool will provide new avenues to study how sperm flagella locally transduce extracellular signals into a specific swimming response.

Targeting optogenetic tools to the primary cilium

To shed light on cAMP signaling in primary cilia, I transferred the optogenetic approach to primary cilia. Our group had shown that optogenetic tools and biosensors can be easily targeted to the sperm flagellum, when being expressed under a sperm-specific promoter (Jansen et al., 2015; Mukherjee et al., 2016; Raju et al., 2019). However, in primary cilia, the IFT machinery and the transition zone tightly control protein import and export (Garcia et al., 2018). Thus, ectopic expression and targeting to primary cilia is commonly realized by fusion to ciliary proteins or parts thereof, such as the GPCRs SSTR3 (Berbari et al., 2008) and 5-HT6 receptor (Moore et al., 2016), the GTPase ARL13B (Jiang et al., 2019), or the first 201 amino acids of the nephrocystin 3 (NPHP3) protein (Mick et al., 2015; Wright et al., 2011). Since using full-length proteins as fusion partners may increase the ciliary abundance of this protein and thereby, may bias the ciliary signaling architecture, I chose to use a fusion to the truncated murine NPHP3 protein (mNphp3(201)), which reportedly lacks functionality (Wright et al., 2011), for ciliary targeting. The optogenetic tools for manipulating cAMP levels, the photo-activated adenylyl cyclase bPAC and the light-activated phosphodiesterase LAPD, however, need to undergo conformational changes to get activated (Gasser et al., 2014; Lindner et al.,

2017) – I observed that the N-terminal fusion of mNphp3(201) impaired bPAC and LAPD activation (Hansen et al., 2020). Thus, I developed a new approach for ciliary targeting to circumvent this problem: I fused nanobodies that bind common fluorescent protein tags like eGFP or mCherry to mNphp3(201) and co-expressed them with fluorescent-protein-tagged proteins to ferry them to the primary cilium (Fig. 3A). bPAC and LAPD functionally tolerate the C-terminal mCherry tag. Co-expressing bPAC-mCherry or LAPD-mCherry with a mNphp3(201)-linked anti-mCherry nanobody, resulted in transport of bPAC or LAPD into cilia (Fig 3B-C), without affecting bPAC or LAPD functionality. Of note, we showed that this approach is also applicable in vivo, in zebrafish (Fig. 3D-E). With the nanobody-based targeting approach, we generated a new flexible targeting approach for fluorescent-protein-tagged proteins into cilia.

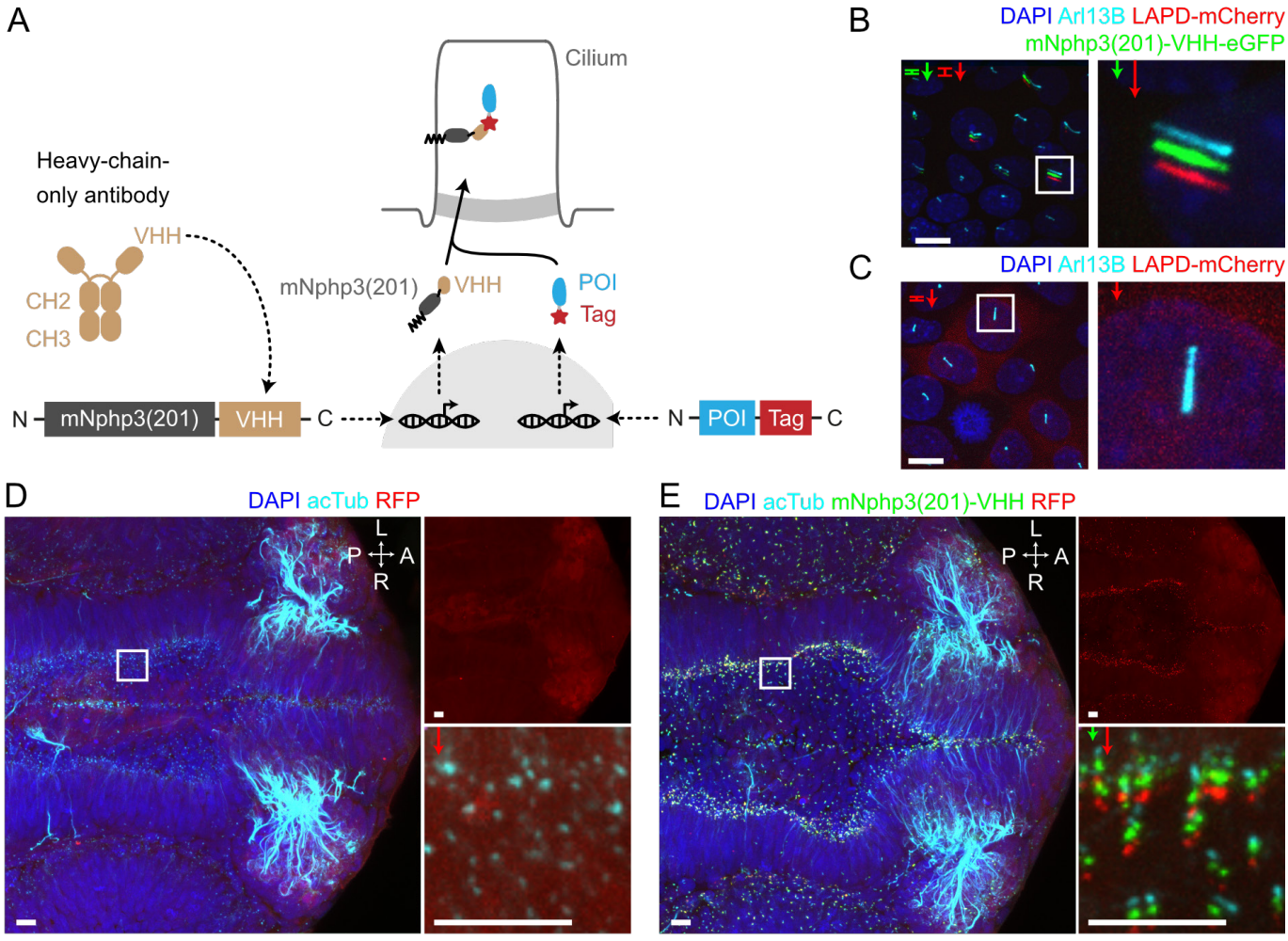

Figure 3: Targeting tagged proteins to the primary cilium using nanobodies. (A) The targeting approach: Nanobodies (VHH) are fused to the C terminus of mNphp3(201) to localize them to the cilium. The protein of interest (POI) is fused to a tag that is recognized by the nanobody. Co-expressing both constructs results in nanobody binding to the tag and, in turn, transport of the POI into the cilium. **(B-C)** Murine inner medullar collecting ducts cells (mIMCD-3 cells), transfected with **(B)** mNphp3(201)-VHH-eGFP and LAPD-mCherry or **(C)** LAPD-mCherry only, stained with an Arl13B antibody to label cilia and DAPI to label DNA. LAPD-mCherry localizes to the cytosol and is transported to the cilium only upon co-expression of the mNphp3(201)-VHH-eGFP construct. **(D)** Neural tube of an Ubi:zebrabow (Pan et al., 2013) transgenic embryo, which expresses RFP, stained with an acetylated-Tubulin antibody to label cilia and DAPI to label nuclei. **(E)** Neural tube of a Ubi:zebrabow (Pan et al., 2013) transgenic embryo, injected with mRNA of an anti-mCherry mNphp3(201)-VHH-eGFP nanobody construct. Scale bars: 10 µm (B-C) or 20 µm (D-E, magnified view: 10 µm). Boxes indicate the position of the magnified views shown at the (bottom) right as inset. Arrows in different colors indicate the direction and the length of the shift of the respective fluorescence channel. The upper right panel in D and E shows the RFP channel only, the bottom right panel shows the magnified view. A: anterior, P: posterior, L: left, R: right. Figures modified from Hansen et al., eLife, 2020, available at https://doi.org/10.7554/eLife.57907, published under CC-BY 4.0 license (https://creativecommons.org/licenses/by/4.0/).

Developing CiliaQ, an image analysis method to study ciliary signaling and morphology

The analysis of primary cilia signaling is image-based and requires image analysis approaches that allow to spatially separate ciliary signals from the cell body. Since such a tool was missing in the primary cilia field like in the sperm flagella field, I also developed an ImageJ plugin for primary cilia: CiliaQ analyzes 2D, 3D, and time-lapse 3D fluorescence microscopy images of cilia with high through-put and determines a comprehensive panel of parameters describing ciliary morphology and (sub)ciliary intensity (Hansen et al., 2021b).

Revealing a cAMP signalosome in primary cilia that drives gene expression and kidney cyst formation

With the optogenetic approach and CiliaQ, I had set the stage to analyze the mechanisms and functions of cAMP signaling in primary cilia. I selected murine epithelial cells from the kidney as a cellular model (murine Inner Medullary Collecting Duct Cells 3 (mIMCD-3)), a standard model to study primary cilia. The role of primary cilia in the kidney is particularly interesting since several ciliopathies, genetic diseases related to ciliary dysfunction, present with kidney cysts (Ma, 2021). One of those diseases, autosomal dominant polycystic kidney disease (ADPKD), is the most common ciliopathy, affecting approximately 1:2000 people (Solazzo et al., 2018). However, the pathomechanism of cystogenesis has been unknown. Similarly, physiological roles of primary cilia in the kidney are little understood – primary cilia in the kidney are likely involved in the sensation of fluid flow albeit the mechanism is unclear (Verschuren et al., 2020).

ADPKD results from mutations in PKD1 or PKD2, encoding polycystin 1 (PC1) or polycystin 2 (PC2), respectively (Pazour et al., 2002; Yoder et al., 2002), which both localize to primary cilia where they form a cation channel complex (Qian et al., 1997; Shen et al., 2016). Some hints pointed to a dysregulation of cAMP signaling in ADPKD: (1) Increased tissue levels of cAMP were detected in the kidneys of ADPKD mouse models (Aguiari et al., 2012; Gattone et al., 2003; Mochizuki et al., 1996; Smith et al., 2006; Starremans et al., 2008; Yamaguchi et al., 1997), (2) Tolvaptan, the only ADPKD drug treatment available, is designed to reduce cAMP levels (Blair, 2019), (3) pharmacologically increasing cAMP levels results in cysts in an in-vitro model and in ex-vivo organoid cultures (Elberg et al., 2012; Hwang et al., 2017), (4) the adenylyl cyclases ADCY5 and ADCY6 localize to the cilium of kidney epithelial cells and knock-out of ADCY5 or ADCY6 reduces cyst formation in ADPKD mouse models (Rees et al., 2014; Wang et al., 2018). It has been proposed that mutations in PKD1 or PKD2 may reduce ciliary calcium levels, which, in turn, could increase ciliary cAMP levels by relieving ADCY5 and ADCY6 from calcium-dependent inhibition (Nachury and Mick, 2019).

I aimed to disentangle the role of cAMP in kidney cyst formation (Hansen et al., 2022). To this end, I established a previously described 3D culture model for cyst formation based on kidney epithelial cells (Elberg et al., 2012) and combined it to the optogenetic approach to manipulate ciliary cAMP levels. I observed that cAMP stimulation through bPAC activation in the cilium (cilia-bPAC) and not in the cytoplasm (cyto-bPAC) triggered cystogenesis (Fig. 4A-B) (Hansen et al., 2022). Combining the optogenetic approach with RNA-sequencing, I could demonstrate that ciliary cAMP signaling evokes a specific gene expression program, distinct from the cell body (Fig. 4C-D). In follow-up experiments, I identified ciliary cAMP/PKA/CREB-dependent regulation of gene expression as a novel concept how the primary cilium controls cellular functions in a specific and spatially distinct manner (Fig. 4E) (Hansen et al., 2022). By decreasing ciliary cAMP levels through activating long PDE4 isoforms with a new small molecule, I could dampen cystogenesis. We confirmed that PDE4 localizes to the primary cilium.

In summary, we provide first mechanistic inside into how ciliary signaling contributes to disease pathogenesis in cystic kidney diseases. Beyond this, our findings also conceptually advance what we know about the biology of primary cilia. So far, sonic hedgehog signaling had been the only well-characterized ciliary signaling pathway. Notably, sonic hedgehog pathway activation involves a reduction in ciliary cAMP levels and ciliary PKA activity, as recently highlighted with an optogenetic approach (Truong et al., 2021). We extend the mechanistic knowledge on ciliary signaling by describing a pathway that involves an increase in ciliary cAMP levels and PKA activity. Thereby, we reveal that ciliary cAMP signaling functions in both directions, via increases and decreases of ciliary cAMP level.

SpermQ, CiliaQ, FreQ are freely available for analysis of cilia images

On a final note, SpermQ (Hansen et al., 2019), CiliaQ (Hansen et al., 2021b), and the multi-focal imaging analysis toolbox (Hansen et al., 2021a) have been developed with the aim to generate methods that can be easily applied by other labs and allow them to gain deep insight from microscopy images without requiring computational skills. I envision that sharing software for image analysis can boost scientific progress. The software, the source code, and the user guides are freely provided online:
https://github.com/hansenjn/SpermQ,
https://github.com/hansenjn/CiliaQ,
https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox.
Recently, we also added another plugin to the family: FreQ (Jeong et al., 2022) (https://github.com/hansenjn/FreQ), which provides an analysis of ciliary beating on densely ciliated cells, based on pixel intensity oscillations.

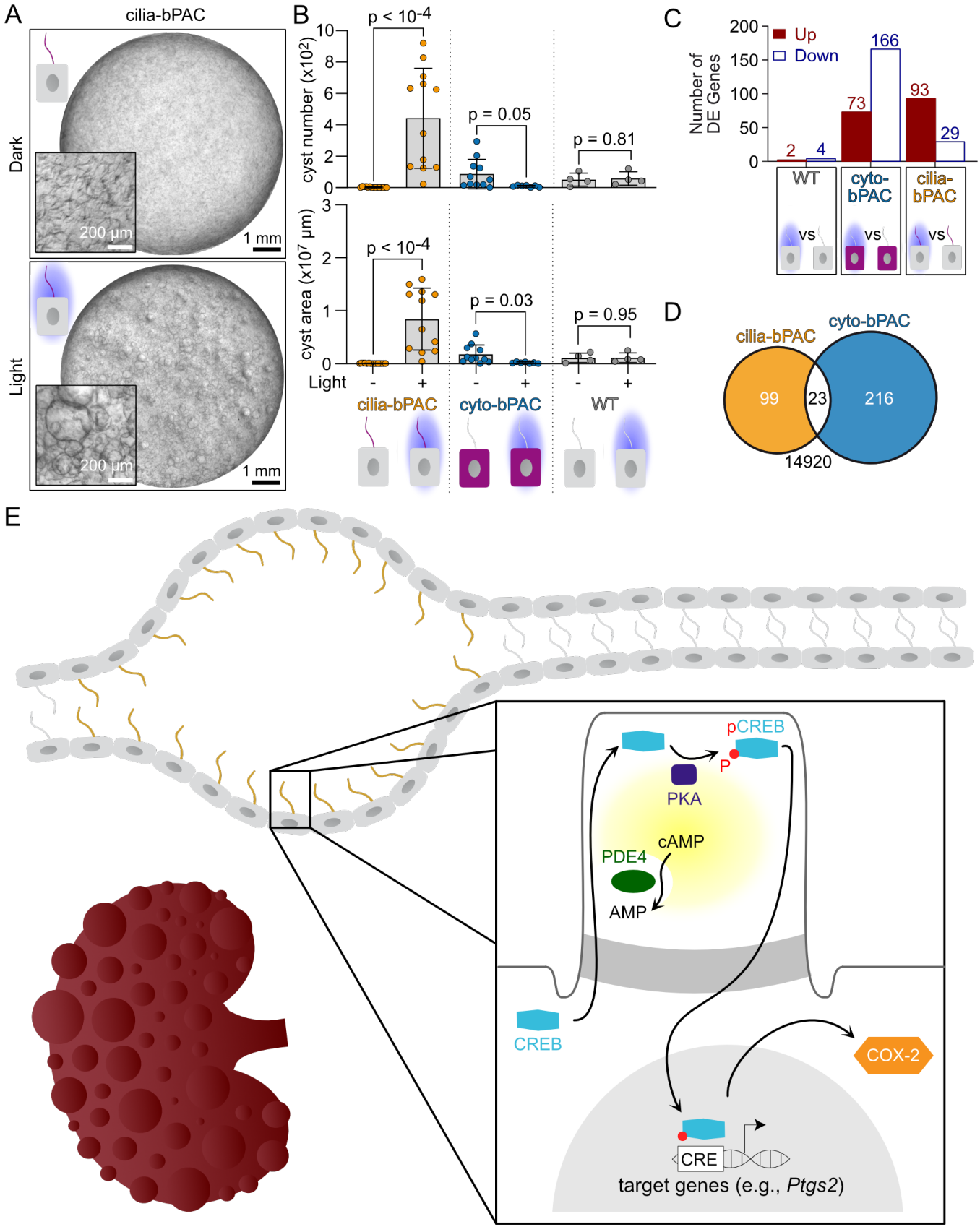
A
cilia-bPAC
Dark
Light
200 µm
1 mm
B
p < 10-4
p = 0.05
p = 0.81
cyst number (x10²)
p < 10-4
p = 0.03
p = 0.95
cyst area (x10⁷ µm)
Light
cilia-bPAC
cyto-bPAC
WT
C
Number of DE Genes
Up
Down
2
4
73
166
93
29
WT
cyto-bPAC
cilia-bPAC
vs
D
cilia-bPAC
cyto-bPAC
99
23
216
14920
E
pCREB
P
PKA
cAMP
PDE4
AMP
CREB
COX-2
CRE
target genes (e.g., Ptgs2)

Figure 4. Using subcellular optogenetics to reveal the role of ciliary cAMP signaling in kidney cells. (A) Murine inner medullary collecting duct cells (mIMCD-3 cells) expressing the photo-activated adenylyl cyclase bPAC targeted to the primary cilium (cilia-bPAC), cultured in 3D for 9 days, in the dark or light (1 h light/1 h dark cycling, 465 nm, 38.8 µW/cm²). In the dark, cilia bPAC cells formed tubular structures, whereas, upon chronic light-stimulation, they formed cysts. **(B)** Quantification of experiments exemplified in A. Cells expressing bPAC targeted to the primary cilium (cilia-bPAC) or to the cell body (cyto-bPAC) and wild-type (WT) cells were analyzed. Only cilia-bPAC cells formed cysts upon light exposure. Data are shown as mean ± SD, each datapoint corresponds to an independent experiment, p-values calculated using an unpaired, two-sided Student's t-test are indicated. **(C)** Optogenetic RNA-Sequencing experiment with cilia-bPAC, cyto-bPAC, and WT cells. For each cell type, the up- or down-regulated genes in cells stimulated by light (1 h, 465 nm, 38.8 µW/cm²) compared to cells kept in the dark were determined. **(D)** Venn diagram of the DE Genes shown in C for cilia-bPAC and cyto-bPAC cells. **(E)** Follow-up experiments revealed a ciliary cAMP signalosome consisting of cAMP/PKA/CREB, which controls a specific gene expression program and, upon chronic stimulation, drives cyst formation. PDE4 isoforms limit the signalosome. Figures modified from Hansen et al., EMBO Rep., 2022, available at https://dx.doi.org/10.15252/embr.202154315, published under CC-BY 4.0 license (https://creativecommons.org/licenses/by/4.0/).

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References

Aguiari, G., F. Bizzarri, A. Bonon, A. Mangolini, E. Magri, M. Pedriali, P. Querzoli, S. Somlo, P.C. Harris, L. Catizone, and L. Del Senno. 2012. Polycystin-1 regulates amphiregulin expression through CREB and AP1 signalling: implications in ADPKD cell proliferation. *J. Mol. Med. (Berl.).* 90:1267-1282.

Alvarez, L., B.M. Friedrich, G. Gompper, and U.B. Kaupp. 2014. The computational sperm cell. *Trends Cell Biol.* 24:198-207.

Anvarian, Z., K. Mykytyn, S. Mukhopadhyay, L.B. Pedersen, and S.T. Christensen. 2019. Cellular signalling by primary cilia in development, organ function and disease. Nat. Rev. *Nephrol.* 15:199-219.

Balbach, M., V. Beckert, J.N. Hansen, and D. Wachten. 2018. Shedding light on the role of cAMP in mammalian sperm physiology. *Mol. Cell. Endocrinol.* 468:111-120.

Baskaran, S., R. Finelli, A. Agarwal, and R. Henkel. 2021. Diagnostic value of routine semen analysis in clinical andrology. *Andrologia.* 53:e13614.

Berbari, N.F., A.D. Johnson, J.S. Lewis, C.C. Askwith, and K. Mykytyn. 2008. Identification of ciliary localization sequences within the third intracellular loop of G protein-coupled receptors. *Mol. Biol. Cell.* 19:1540-1547.

Blair, H.A. 2019. Tolvaptan: A Review in Autosomal Dominant Polycystic Kidney Disease. *Drugs.* 79:303-313.

Bukatin, A., I. Kukhtevich, N. Stoop, J. Dunkel, and V. Kantsler. 2015. Bimodal rheotactic behavior reflects flagellar beat asymmetry in human sperm cells. *Proc. Natl. Acad. Sci. U. S. A.* 112:15904-15909.

Dardikman-Yoffe, G., S.K. Mirsky, I. Barnea, and N.T. Shaked. 2020. High-resolution 4-D acquisition of freely swimming human sperm cells without staining. *Sci. Adv.* 6:eaay7619.

Elberg, D., M.A. Turman, N. Pullen, and G. Elberg. 2012. Prostaglandin E2 stimulates cystogenesis through EP4 receptor in IMCD-3 cells. *Prostaglandins Other Lipid Mediat.* 98:11-16.

Gadadhar, S., G. Alvarez Viar, J.N. Hansen, A. Gong, A. Kostarev, C. Ialy-Radio, S. Leboucher, M. Whitfield, A. Ziyyat, A. Touré, L. Alvarez, G. Pigino, and C. Janke. 2021. Tubulin glycylation controls axonemal dynein activity, flagellar beat, and male fertility. *Science.* 371:eabd4914.

Gallagher, M.T., G. Cupples, E.H. Ooi, J.C. Kirkman-Brown, and D.J. Smith. 2019. Rapid sperm capture: high-throughput flagellar waveform analysis. *Hum. Reprod.* 34:1173-1185.

Garcia, G., 3rd, D.R. Raleigh, and J.F. Reiter. 2018. How the Ciliary Membrane Is Organized Inside-Out to Communicate Outside-In. *Curr. Biol.* 28:R421-R434.

Gasser, C., S. Taiber, C.M. Yeh, C.H. Wittig, P. Hegemann, S. Ryu, F. Wunder, and A. Moglich. 2014. Engineering of a red-light-activated human cAMP/cGMP-specific phosphodiesterase. *Proc. Natl. Acad. Sci. U. S. A.* 111:8803-8808.

Gattone, V.H., 2nd, X. Wang, P.C. Harris, and V.E. Torres. 2003. Inhibition of renal cystic disease development and progression by a vasopressin V2 receptor antagonist. *Nat. Med.* 9:1323-1326.

Gong, A., S. Rode, G. Gompper, U.B. Kaupp, J. Elgeti, B.M. Friedrich, and L. Alvarez. 2021. Reconstruction of the three-dimensional beat pattern underlying swimming behaviors of sperm. *Eur. Phys. J. E. Soft Matter.* 44:87.

Hansen, J., S. Rassmann, J. Jikeli, and D. Wachten. 2019. SpermQ–A Simple Analysis Software to Comprehensively Study Flagellar Beating and Sperm Steering. *Cells.* 8:10.

Hansen, J.N., A. Gong, D. Wachten, R. Pascal, A. Turpin, J.F. Jikeli, U.B. Kaupp, and L. Alvarez. 2021a. Multifocal imaging for precise, label-free tracking of fast biological processes in 3D. *Nature communication.* 12:4574.

Hansen, J.N., F. Kaiser, C. Klausen, B. Stuven, R. Chong, W. Bonigk, D.U. Mick, A. Moglich, N. Jurisch-Yaksi, F.I. Schmidt, and D. Wachten. 2020. Nanobody-directed targeting of optogenetic tools to study signaling in the primary cilium. *eLife.* 9:e57907.

Hansen, J.N., F. Kaiser, P. Leyendecker, B. Stuven, J.H. Krause, F. Derakhshandeh, J. Irfan, T.J. Sroka, K.M. Preval, P.B. Desai, M. Kraut, H. Theis, A.D. Drews, E. De-Domenico, K. Handler, G.J. Pazour, D.J.P. Henderson, D.U. Mick, and D. Wachten. 2022. A cAMP signalosome in primary cilia drives gene expression and kidney cyst formation. *EMBO Rep.* 23:e54315.

Hansen, J.N., S. Rassmann, B. Stuven, N. Jurisch-Yaksi, and D. Wachten. 2021b. CiliaQ: a simple, open-source software for automated quantification of ciliary morphology and fluorescence in 2D, 3D, and 4D images. *Eur. Phys. J. E Soft Matter.* 44:18.

Hwang, V.J., X. Zhou, X. Chen, J. Trott, O. Abu Aboud, K. Shim, L.K. Dionne, K.J. Chmiel, W. Senapedis, E. Baloglu, M.R. Mahjoub, X. Li, and R.H. Weiss. 2017. Anticystogenic activity of a small molecule PAK4 inhibitor may be a novel treatment for autosomal dominant polycystic kidney disease. *Kidney Int.* 92:922-933.

Ishimoto, K., H. Gadelha, E.A. Gaffney, D.J. Smith, and J. Kirkman-Brown. 2017. Coarse-Graining the Fluid Flow around a Human Sperm. *Phys. Rev. Lett.* 118:124501.

Jansen, V., L. Alvarez, M. Balbach, T. Strunker, P. Hegemann, U.B. Kaupp, and D. Wachten. 2015. Controlling fertilization and cAMP signaling in sperm by optogenetics. *eLife.* 4.

Jeong, I., J.N. Hansen, D. Wachten, and N. Jurisch-Yaksi. 2022. Measurement of ciliary beating and fluid flow in the zebrafish adult telencephalon. *STAR Protocols.* 3:101542.

Jiang, J.Y., J.L. Falcone, S. Curci, and A.M. Hofer. 2019. Direct visualization of cAMP signaling in primary cilia reveals up-regulation of ciliary GPCR activity following Hedgehog activation. *Proc. Natl. Acad. Sci. U. S. A.* 116:12066-12071.

Lindner, R., E. Hartmann, M. Tarnawski, A. Winkler, D. Frey, J. Reinstein, A. Meinhart, and I. Schlichting. 2017. Photoactivation Mechanism of a Bacterial Light-Regulated Adenylyl Cyclase. *J. Mol. Biol.* 429:1336-1351.

Ma, M. 2021. Cilia and polycystic kidney disease. *Semin. Cell Dev. Biol.* 110:139-148.

Mick, D.U., R.B. Rodrigues, R.D. Leib, C.M. Adams, A.S. Chien, S.P. Gygi, and M.V. Nachury. 2015. Proteomics of Primary Cilia by Proximity Labeling. *Dev. Cell.* 35:497-512.

Miki, K., and D.E. Clapham. 2013. Rheotaxis guides mammalian sperm. *Curr. Biol.* 23:443-452.

Mochizuki, T., G. Wu, T. Hayashi, S.L. Xenophontos, B. Veldhuisen, J.J. Saris, D.M. Reynolds, Y. Cai, P.A. Gabow, A. Pierides, W.J. Kimberling, M.H. Breuning, C.C. Deltas, D.J. Peters, and S. Somlo. 1996. PKD2, a gene for polycystic kidney disease that encodes an integral membrane protein. *Science.* 272:1339-1342.

Moore, B.S., A.N. Stepanchick, P.H. Tewson, C.M. Hartle, J. Zhang, A.M. Quinn, T.E. Hughes, and T. Mirshahi. 2016. Cilia have high cAMP levels that are inhibited by Sonic Hedgehog-regulated calcium dynamics. *Proc. Natl. Acad. Sci. U. S. A.* 113:13069-13074.

Mukherjee, S., V. Jansen, J.F. Jikeli, H. Hamzeh, L. Alvarez, M. Dombrowski, M. Balbach, T. Strunker, R. Seifert, U.B. Kaupp, and D. Wachten. 2016. A novel biosensor to study cAMP dynamics in cilia and flagella. *eLife.* 5.

Muschol, M., C. Wenders, and G. Wennemuth. 2018. Four-dimensional analysis by high-speed holographic imaging reveals a chiral memory of sperm flagella. *PloS one.* 13:e0199678.

Nachury, M.V., and D.U. Mick. 2019. Establishing and regulating the composition of cilia for signal transduction. *Nat. Rev. Mol. Cell Biol.* 20:389-405.

Olstad, E.W., C. Ringers, J.N. Hansen, A. Wens, C. Brandt, D. Wachten, E. Yaksi, and N. Jurisch-Yaksi. 2019. Ciliary Beating Compartmentalizes Cerebrospinal Fluid Flow in the Brain and Regulates Ventricular Development. *Curr. Biol.* 29:229-241 e226.

Pan, Y.A., T. Freundlich, T.A. Weissman, D. Schoppik, X.C. Wang, S. Zimmerman, B. Ciruna, J.R. Sanes, J.W. Lichtman, and A.F. Schier. 2013. Zebrabow: multispectral cell labeling for cell tracing and lineage analysis in zebrafish. *Development.* 140:2835-2846.

Pazour, G.J., J.T. San Agustin, J.A. Follit, J.L. Rosenbaum, and G.B. Witman. 2002. Polycystin-2 localizes to kidney cilia and the ciliary level is elevated in orpk mice with polycystic kidney disease. *Curr. Biol.* 12:R378-380.

Qian, F., F.J. Germino, Y. Cai, X. Zhang, S. Somlo, and G.G. Germino. 1997. PKD1 interacts with PKD2 through a probable coiled-coil domain. *Nat. Genet.* 16:179-183.

Raju, D.N., J.N. Hansen, S. Rassmann, B. Stüven, J.F. Jikeli, T. Strünker, H.G. Körschen, A. Möglich, and D. Wachten. 2019. Cyclic Nucleotide-Specific Optogenetics Highlights Compartmentalization of the Sperm Flagellum into cAMP Microdomains. *Cells.* 8:648.

Rees, S., W. Kittikulsuth, K. Roos, K.A. Strait, A. Van Hoek, and D.E. Kohan. 2014. Adenylyl cyclase 6 deficiency ameliorates polycystic kidney disease. *J. Am. Soc. Nephrol.* 25:232-237.

Shen, P.S., X. Yang, P.G. DeCaen, X. Liu, D. Bulkley, D.E. Clapham, and E. Cao. 2016. The Structure of the Polycystic Kidney Disease Channel PKD2 in Lipid Nanodiscs. *Cell.* 167:763-773 e711.

Smith, L.A., N.O. Bukanov, H. Husson, R.J. Russo, T.C. Barry, A.L. Taylor, D.R. Beier, and O. Ibraghimov-Beskrovnaya. 2006. Development of polycystic kidney disease in juvenile cystic kidney mice: insights into pathogenesis, ciliary abnormalities, and common features with human disease. *J. Am. Soc. Nephrol.* 17:2821-2831.

Solazzo, A., F. Testa, S. Giovanella, M. Busutti, L. Furci, P. Carrera, M. Ferrari, G. Ligabue, G. Mori, M. Leonelli, G. Cappelli, and R. Magistroni. 2018. The prevalence of autosomal dominant polycystic kidney disease (ADPKD): A meta-analysis of European literature and prevalence evaluation in the Italian province of Modena suggest that ADPKD is a rare and underdiagnosed condition. *PloS one.* 13:e0190430.

Starremans, P.G., X. Li, P.E. Finnerty, L. Guo, A. Takakura, E.G. Neilson, and J. Zhou. 2008. A mouse model for polycystic kidney disease through a somatic in-frame deletion in the 5' end of Pkd1. *Kidney Int.* 73:1394-1405.

Truong, M.E., S. Bilekova, S.P. Choksi, W. Li, L.J. Bugaj, K. Xu, and J.F. Reiter. 2021. Vertebrate cells differentially interpret ciliary and extraciliary cAMP. *Cell.* 184:2911-2926 e2918.

Verschuren, E.H.J., C. Castenmiller, D.J.M. Peters, F.J. Arjona, R.J.M. Bindels, and J.G.J. Hoenderop. 2020. Sensing of tubular flow and renal electrolyte transport. *Nat. Rev. Nephrol.* 16:337-351.

Wachten, D., J.F. Jikeli, and U.B. Kaupp. 2017. Sperm Sensory Signaling. Cold Spring Harb. *Perspect. Biol.* 9.

Wang, Q., P. Cobo-Stark, V. Patel, S. Somlo, P.L. Han, and P. Igarashi. 2018. Adenylyl cyclase 5 deficiency reduces renal cyclic AMP and cyst growth in an orthologous mouse model of polycystic kidney disease. *Kidney Int.* 93:403-415.

Wright, K.J., L.M. Baye, A. Olivier-Mason, S. Mukhopadhyay, L. Sang, M. Kwong, W. Wang, P.R. Pretorius, V.C. Sheffield, P. Sengupta, D.C. Slusarski, and P.K. Jackson. 2011. An ARL3-UNC119-RP2 GTPase cycle targets myristoylated NPHP3 to the primary cilium. *Genes Dev.* 25:2347-2360.

Yamaguchi, T., S. Nagao, M. Kasahara, H. Takahashi, and J.J. Grantham. 1997. Renal accumulation and excretion of cyclic adenosine monophosphate in a murine model of slowly progressive polycystic kidney disease. *Am. J. Kidney Dis.* 30:703-709.

Yoder, B.K., X. Hou, and L.M. Guay-Woodford. 2002. The polycystic kidney disease proteins, polycystin-1, polycystin-2, polaris, and cystin, are co-localized in renal cilia. *J. Am. Soc. Nephrol.* 13:2508-2516.

About the author

Jan has always been fascinated by interdisciplinary approaches between computer science, physics, biology, and medicine. His interest in computer sciences emerged during childhood when he started learning java programming. Later, Jan studied Molecular Biomedicine (Bachelor of Science) and Life and Medical Sciences (Master of Science) at the University of Bonn, supported with a scholarship by the Studienstiftung des deutschen Volkes e.V. Since beginning of his studies, he develops image analysis software for biologists, starting with MotiQ, a software to quantify cell morphology and motility of microglia. With his Master thesis at the groups of Prof. Dr. U. Benjamin Kaupp (caesar, Bonn, Germany) and Prof. Dr. Dagmar Wachten (now: University of Bonn, Germany), Jan got into the cilia field. At the end of his master studies, Jan joined the Yaksi lab at the Kavli Institute for Systems Neuroscience (NTNU, Norway) as an intern to work with Dr. Nathalie Jurisch-Yaksi on understanding the coordination of motile cilia in the olfactory pit of zebrafish larvae. In his PhD project in the lab of Prof. Dr. Dagmar Wachten, Jan applied a combination of 3D cell culture, optogenetics, molecular biology techniques, transcriptomics, advanced microscopy, and image analysis, to shed light on cAMP signaling in cilia. As a PhD student, Jan was supported with a PhD fellowship by the Boehringer Ingelheim Fonds. Jan now started his next project, at the group of Prof. Dr. Emma Lundberg (KTH – Royal Institute of Technology, Sweden and Stanford Bioengineering / Stanford Pathology, USA), supported with a postdoc fellowship by the Wenner-Gren Foundations, Sweden. He now studies human cilia and flagella from a systems biology perspective, using large-scale antibody-based spatial proteomics and big data analysis approaches.

BINDER INNOVATION PRIZE

Mitochondria-Microbe Conflict

Lena Pernas

Summary:
Organelles change their behavior upon infection with diverse pathogens, changes that have long been considered to be microbe-driven (Escoll *et al.*, 2016). Our past and current work challenges this perception and instead proposes that these changes – at least in part – comprise a host response that counteracts microbes (Li *et al.*, 2022; Pernas *et al.*, 2018). Here, I summarize our work addressing a host-pathogen arms race derived from metabolic conflict between the host-cell mitochondria and the human parasite *Toxoplasma gondii*, which infects an estimated 1/3 of the population.

Domesticated microbe vs. invading microbe

Mitochondria regulate a myriad of cellular functions, but in the context of infection these dynamic organelles are considered static bystanders (Fig. 1). However, to generate energy, mitochondria consume nutrients that invading microbes depend on. This competing interest predicts an inverse relationship between mitochondrial health and microbial fitness. Although several pathogens disrupt host mitochondrial function, whether mitochondria act to impede pathogen replication was unknown.

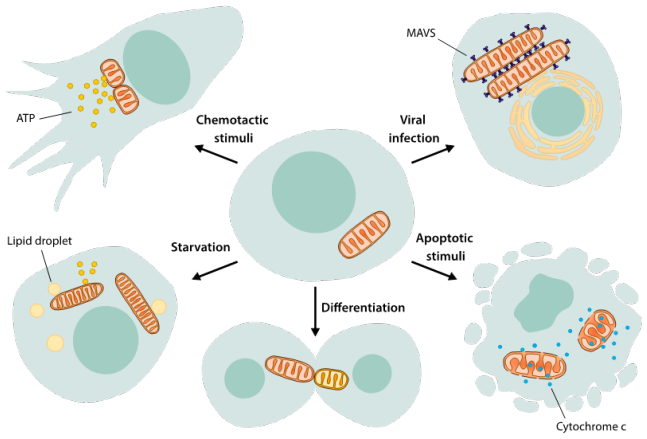

Figure 1: Mitochondrial dynamics mediate diverse cellular processes (from (Pernas and Scorrano, 2016)

Interestingly, these organelles rapidly change their behavior during microbial infection. One of the earliest described changes is mitochondrial trafficking to and surrounding of vacuoles in which parasites reside and replicate (Jones and Hirsch, 1972)(Fig. 2). Although this phenomenon was first discovered in murine macrophages infected with the apicomplexan parasite *Toxoplasma gondii* in 1972, this interaction has since been shown to occur in a wide range of host cell species including *Hartmanella vermiformis* (amoeba) and Homo sapiens, and with several evolutionarily divergent prokaryotic and eukaryotic pathogens including the bacteria *Legionella pneumophila* and Chlamydia *psitacci* (Dumoux and Hayward, 2016). Such changes in mitochondrial distribution were long considered to represent a microbial attempt to siphon ATP, or escape recognition by the endolysosomal system despite the lack of clear evidence to support either scenario.

An observation we made a few years ago reframed how we perceived the changes in mitochondrial behavior. We noticed that mitochondria surrounding the parasite vacuole were elongated in comparison to mitochondria in uninfected cells; changes in mitochondrial shape are well known to underly changes in mitochondrial function (Blank *et al.*, 2018; Pernas *et al.*, 2014; Pernas and Scorrano, 2016). This led us to consider if the changes in mitochondrial morphology during infection reflected a host response to counteract *Toxoplasma* – rather than parasite exploitation of host mitochondria.

Because invading microbes occupy the host cytosol and take up nutrients on which host organelles such as mitochondria also depend, we tested the possibility that the elongation of mitochondria during infection reflected changes in their metabolism that were detrimental to *Toxoplasma*. We found that *Toxoplasma* coopts host cell lipophagy to deplete fatty acids from lipid droplets, their storage site in the cell, and obtain fatty acids (FAs) needed for proliferation. To counter *Toxoplasma* nutrient siphoning, mitochondrial elongate, a response that enhances mitochondrial FA uptake and limits FA acquisition by *Toxoplasma*, thereby limiting the growth of the parasite. The pharmacological modulation of mitochondrial FA metabolism profoundly affected parasite proliferation; a small molecule activator of fatty acid uptake by mitochondria restricted parasite growth, while

an inhibitor promoted parasite growth (Pernas *et al.*, 2018). Thus, by regulating parasite access to FAs, mitochondrial control parasite growth. These results demonstrate that mitochondrial metabolism can be rewired to act a first line of defense against an intracellular microbe.

Keep your enemies closer

Host mechanisms to impede the exploitation of nutrients by microbes would be expected to lead to the emergence to microbial resistance mechanisms. Interestingly, a hallmark of the encasement of microbial niches such as the *Toxoplasma* vacuole by mitochondria is the formation of regions of close membrane apposition between the host outer mitochondrial membrane (OMM) and the parasite vacuole membrane (PVM)(Sinai *et al.*, 1997)(Fig. 2). These regions are less than 30 nm in distance, between 2-10µm in length and can be referred to as trans-kingdom 'contact sites,' a term coined to describe homotypic or heterotypic regions of membrane association between organelles (Scorrano *et al.*, 2019). Since the initial discovery of host mitochondrial association (HMA) in macrophages infected with *Toxoplasma* in 1972, the question of what factors, host or parasite, mediates this association has been of interest. Previous work suggested that *Toxoplasma* HMA was mediated by *Toxoplasma* rhoptry protein 2 (ROP2)(Sinai and Joiner, 2001). However, in subsequent work, we showed that parasites lacking ROP2 expression were indistinguishable from wild-type parasites in their ability to recruit host mitochondria, reopening the question of what parasite factors mediated HMA (Pernas and Boothroyd, 2010).

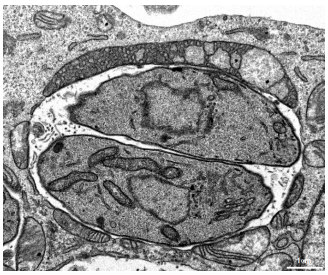

Figure 2: Mitochondria surround and form contact sites with the parasite vacuole.

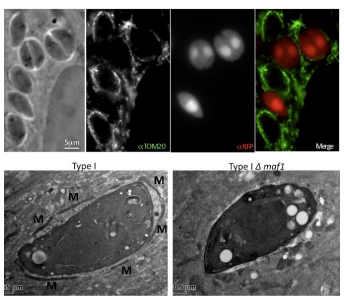

Figure 3: Wild-type Toxoplasma are surrounded by mitochondria and thus positive for host-mitochondrial association (HMA), but *Maf1* knockout parasites (top, in red, bottom on right)) are not; from Pernas *et al.*, 2014

Given the expanded understanding of the diverse roles performed by mitochondria (at the time, we had not yet discovered that mitochondrial metabolism restricts parasite growth *Toxoplasma*), we hypothesized that HMA conferred a selective advantage to the parasites. We found that HMA in *Toxoplasma* is mediated by a novel, secreted parasite effector that we named mitochondrial association factor 1 (MAF1). We established that MAF1 is necessary and sufficient for HMA, and showed that HMA is associated with substantially altered levels of cytokines during *Toxoplasma* infection *in vitro* and in vivo (Pernas *et al.*, 2014) (Fig. 3). These results demonstrate that HMA represents a strategy by which a microbe can interface with its host.

Host-pathogen arms race derived from metabolic competition

That *Toxoplasma* secretes an effector protein to tether mitochondria contradicted our model. Why would a pathogen want to bring its nutrient competitor closer in? An answer came from monitoring cells at late stages of infection (and thus in cells in which the parasites 'won' as they proliferated), where mitochondria were fragmented. Because mitochondrial fragmentation is often linked with metabolic dysfunction and occurs during late stages of infection with several other pathogens including Legionella, we hypothesized these changes at later stages of infection reflected *Toxoplasma*'s response to mitochondrial metabolic defenses (Escoll *et al.*, 2017; Pernas *et al.*, 2018).

To more closely study how *Toxoplasma* impacted host mitochondria, we focused on the outer mitochondrial membrane (OMM), the compartment of mitochondria that is exposed to and in contact with the parasite vacuole during infection. We found that mitochondria in contact with the *Toxoplasma* vacuole released large structures that were positive for OMM but lacked markers of other mitochondrial compartments, which we termed 'SPOTs.' Having identified *Toxoplasma* infection induced OMM remodeling, we next dissected how these structures are formed. We found that the interaction between the secreted effector protein TgMAF1 – which tethers mitochondria to the parasite vacuole – and the host mitochondrial receptor TOM70 was required for SPOT formation. TgMAF1 led to the degradation of the proteins that mediate mitochondrial nutritional defense during infection. Although we are still working to elucidate the underlying mechanism, our results support a model in which TgMAF1 mimics a mitochondrial protein to hijack a host stress response to counteract mitochondrial nutrient competition (Fig. 4). Host TOM70 on associated mitochondria bind TgMAF1 and attempts to import it. However, because TgMAF1 is integrated into the parasite vacuole membrane, it cannot be imported and thus causes a constitutive stress at the OMM and its shedding (Li *et al.*, 2022). In support of this model, the overexpression of an OMM protein independently of infection, also induces the shedding of structures that look like SPOTs.

Future Directions:

Collectively, our work reveals a host-pathogen arms race derived from metabolic conflict between *Toxoplasma* and host-cell mitochondria. Our work also showcases the utility of using microbial infection as a lens to study mitochondria.

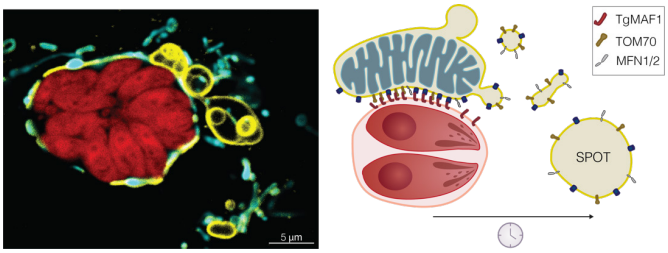

Figure 4: : Mitochondria lose shed SPOTs positive for mitochondrial membrane (OMM; yellow) but not matrix (cyan) during the course of infection in a manner dependent on TgMAF1 and TOM70.

Using this lens, we would like to address: 1) How do organelles sense and respond to microbes? Although we have uncovered one mechanism by which mitochondria actively defend against microbes, we do not know how these organelles, or any organelle for that matter, sense the presence of a microbe and alter their function accordingly. 2) What other metabolic mechanisms enable cells to restrict pathogen growth? My recent work and that of others has shown that mitochondrial metabolism serves as an innate immune defense mechanism to restrict microbial growth. However, the mechanisms that mediate changes in cellular metabolism during infection and the extent to which they regulate microbial growth are largely uncharacterized. In a previous review, we have outlined potential strategies of metabolic defense that we will investigate going forward (Pernas, 2021). 3) Do mitochondria also mediate mutualistic relationships between host and microbe? Several neurotropic pathogens including *Toxoplasma* establish chronic infections that last for the lifetime of the host. To establish a chronic infection and thus succeed in long-term colonization of a host, *Toxoplasma* faces many challenges including establishing residence in a long-lived host cell. How *Toxoplasma* accomplishes such a feat is unclear. Although chronic infections are simplified often as 'avirulent' infection because they do not cause overt disease, the possibility remains that these microbes may transition from an acute antagonistic relationship, to a chronic mutualistic symbiosis with their host. Lastly, and perhaps most important, what is the relevance of human metabolism during infection? Several reports suggest that dietary intake strongly influences human susceptibility to infectious diseases. Children infected with malaria and fed with carbohydrates had increased fever cycles (Murray *et al.*, 1978), raising the question of whether these carbohydrates translate into an added energy source or growth signal for parasites (Mancio-Silva *et al.*, 2017). Metabolic disorders have emerged as major risk factors for microbial infection in humans. Defects in insulin signaling cause hyperglycemia and an array of subsequent metabolic changes that are highly associated with bacterial infection (Casqueiro *et al.*, 2012; Joshi *et al.*, 1999). Inborn errors of mitochondrial metabolism are also known to predispose children to more recurrent microbial infections (Gessner *et al.*, 2013; Walker *et al.*, 2014a; Walker *et al.*, 2014b). A routine examination of infection history in addition to metabolic profiling (i.e. blood glucose and lipid levels) in patients with inborn errors of metabolism or metabolic disorders will likely reveal novel risk factors for infectious disease, as will studies addressing the impact of diet and metabolic status on the progression of microbial infection.

In conclusion, the metabolism of a host cell should not simply be perceived as being subverted by intracellular microbes for their nutritional benefit, but as a key executor of host defense during microbial infection. Approaching the host–pathogen interaction from a framework in which host metabolic processes can be rewired to actively suppress pathogen proliferation will increase our knowledge of the mechanisms employed by cells to defend against microbes, and broaden our understanding of how metabolism influences susceptibility to infectious disease.

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References:

Blank, M.L., Parker, M.L., Ramaswamy, R., Powell, C.J., English, E.D., Adomako-Ankomah, Y., Pernas, L.F., Workman, S.D., Boothroyd, J.C., Boulanger, M.J., and Boyle, J.P. (2018). A *Toxoplasma* gondii locus required for the direct manipulation of host mitochondria has maintained multiple ancestral functions. Mol Microbiol. 10.1111/mmi.13947.

Casqueiro, J., Casqueiro, J., and Alves, C. (2012). Infections in patients with diabetes mellitus: A review of pathogenesis. Indian J Endocrinol Metab *16 Suppl 1*, S27-36. 10.4103/2230-8210.94253.

Dumoux, M., and Hayward, R.D. (2016). Membrane contact sites between pathogen-containing compartments and host organelles. Biochim Biophys Acta *1861*, 895-899. 10.1016/j.bbalip.2016.01.018.

Escoll, P., Mondino, S., Rolando, M., and Buchrieser, C. (2016). Targeting of host organelles by pathogenic bacteria: a sophisticated subversion strategy. Nat Rev Microbiol *14*, 5-19. 10.1038/nrmicro.2015.1.

Escoll, P., Song, O.R., Viana, F., Steiner, B., Lagache, T., Olivo-Marin, J.C., Impens, F., Brodin, P., Hilbi, H., and Buchrieser, C. (2017). Legionella pneumophila Modulates Mitochondrial Dynamics to Trigger Metabolic Repurposing of Infected Macrophages. Cell Host Microbe 22, 302-316 e307. 10.1016/j.chom.2017.07.020.

Gessner, B.D., Gillingham, M.B., Wood, T., and Koeller, D.M. (2013). Association of a genetic variant of carnitine palmitoyltransferase 1A with infections in Alaska Native children. J Pediatr *163*, 1716-1721. 10.1016/j.jpeds.2013.07.010.

Jones, T.C., and Hirsch, J.G. (1972). The interaction between *Toxoplasma* gondii and mammalian cells. II. The absence of lysosomal fusion with phagocytic vacuoles containing living parasites. J Exp Med *136*, 1173-1194.

Joshi, N., Caputo, G.M., Weitekamp, M.R., and Karchmer, A.W. (1999). Infections in patients

with diabetes mellitus. N Engl J Med *341*, 1906-1912. 10.1056/NEJM199912163412507.

Li, X., Straub, J., Medeiros, T.C., Mehra, C., den Brave, F., Peker, E., Atanassov, I., Stillger, K., Michaelis, J.B., Burbridge, E., *et al.* (2022). Mitochondria shed their outer membrane in response to infection-induced stress. Science *375*, eabi4343. 10.1126/science.abi4343.

Mancio-Silva, L., Slavic, K., Grilo Ruivo, M.T., Grosso, A.R., Modrzynska, K.K., Vera, I.M., Sales-Dias, J., Gomes, A.R., MacPherson, C.R., Crozet, P., *et al.* (2017). Nutrient sensing modulates malaria parasite virulence. Nature *547*, 213-216. 10.1038/nature23009.

Murray, M.J., Murray, A.B., Murray, N.J., and Murray, M.B. (1978). Diet and cerebral malaria: the effect of famine and refeeding. Am J Clin Nutr *31*, 57-61. 10.1093/ajcn/31.1.57.

Pernas, L. (2021). Cellular metabolism in the defense against microbes. J Cell Sci 134. 10.1242/jcs.252023.

Pernas, L., Adomako-Ankomah, Y., Shastri, A.J., Ewald, S.E., Treeck, M., Boyle, J.P., and Boothroyd, J.C. (2014). *Toxoplasma* effector MAF1 mediates recruitment of host mitochondria and impacts the host response. PLoS Biol *12*, e1001845. 10.1371/journal.pbio.1001845.

Pernas, L., Bean, C., Boothroyd, J.C., and Scorrano, L. (2018). Mitochondria Restrict Growth of the Intracellular Parasite *Toxoplasma* gondii by Limiting Its Uptake of Fatty Acids. Cell Metab *27*, 886-897 e884. 10.1016/j.cmet.2018.02.018.

Pernas, L., and Boothroyd, J.C. (2010). Association of host mitochondria with the parasitophorous vacuole during *Toxoplasma* infection is not dependent on rhoptry proteins ROP2/8. Int J Parasitol *40*, 1367-1371. S0020-7519(10)00249-3 [pii]

10.1016/j.ijpara.2010.07.002.

Pernas, L., and Scorrano, L. (2016). Mito-Morphosis: Mitochondrial Fusion, Fission, and Cristae Remodeling as Key Mediators of Cellular Function. Annu Rev Physiol *78*, 505-531. 10.1146/annurev-physiol-021115-105011.

Scorrano, L., De Matteis, M.A., Emr, S., Giordano, F., Hajnoczky, G., Kornmann, B., Lackner, L.L., Levine, T.P., Pellegrini, L., Reinisch, K., *et al.* (2019). Coming together to define membrane contact sites. Nat Commun *10*, 1287. 10.1038/s41467-019-09253-3.

Sinai, A., Webster, P., and Joiner, K. (1997). Association of host cell endoplasmic reticulum and mitochondria with the *Toxoplasma* gondii parasitophorous vacuole membrane: a high affinity interaction. J Cell Sci *110*, 2117-2128.

Sinai, A.P., and Joiner, K.A. (2001). The *Toxoplasma* gondii protein ROP2 mediates host organelle association with the parasitophorous vacuole membrane. J Cell Biol *154*, 95-108.

Walker, M.A., Slate, N., Alejos, A., Volpi, S., Iyengar, R.S., Sweetser, D., Sims, K.B., and Walter, J.E. (2014a). Predisposition to infection and SIRS in mitochondrial disorders: 8 years' experience in an academic center. J Allergy Clin Immunol Pract *2*, 465-468, 468 e461. 10.1016/j.jaip.2014.02.009.

Walker, M.A., Volpi, S., Sims, K.B., Walter, J.E., and Traggiai, E. (2014b). Powering the immune system: mitochondria in immune function and deficiency. J Immunol Res *2014*, 164309. 10.1155/2014/164309.

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Eppendorf Young Investigator Award Finalist	2022
Science News: Top 10 Scientists to Watch	2017
Life Sciences Research Foundation Fellowship	2015–2018
EMBO LT Fellowship	2014–2015
DTI-IMPORT Fellowship	2014
Gabilan Fellow, Stanford Graduate Fellowship	2008–2013
National Science Foundation Fellowship	2008–2013
Maximizing Access to Research Careers Scholar (NIH)	2006-2008

WERNER RISAU PRIZE

A human brain vascular atlas reveals diverse mediators of Alzheimer's risk

Andrew Chris Yang

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Summary

The human brain vasculature is of vast medical importance: its dysfunction causes disability and death[1], and the specialized structure it forms – the blood-brain barrier – impedes treatment of nearly all brain disorders[2,3]. Yet, we have no molecular map of the human brain vasculature. Here, we develop Vessel Isolation and Nuclei Extraction for Sequencing (VINE-seq) to profile the major human brain vascular and perivascular cell types through 143,793 single-nucleus transcriptomes from 25 hippocampus and cortex samples of 17 cognitively normal and Alzheimer's disease (AD) subjects. We identify brain region- and species-enriched genes and pathways. We reveal molecular principles of human arteriovenous organization, recapitulating a gradual endothelial and punctuated mural cell continuum. We discover two subtypes of human pericytes, marked by solute transport and extracellular matrix (ECM) organization; and define perivascular versus meningeal fibroblast specialization. In AD, we observe selective vulnerability of ECM-maintaining pericytes and gene expression patterns implicating dysregulated blood flow. With an expanded survey of brain cell types, we find 30 of the top 45 AD GWAS genes expressed in the human brain vasculature, confirmed by immunostaining. Vascular GWAS genes map to endothelial protein transport, adaptive immune, and ECM pathways. Many are microglia-specific in mice, suggesting a partial evolutionary transfer of AD risk. Our work unravels the molecular basis of the human brain vasculature, informing our understanding of overall brain health, disease, and therapy.

Main Text

Brain health depends on brain vascular health. The brain is one of the most highly perfused organs in the body, necessary to meet its unique metabolic needs[4]. Brain vascular dysfunction contributes to stroke[1], congenital neurological disorders[5] and age-related neurodegenerative disease[5,6]. The brain vasculature forms a special structure, the blood-brain barrier (BBB), that mediates selective and hemodynamically responsive movement of molecules between the blood and brain[2,3]. While necessary for optimal neuronal function[4], the BBB frustrates the pharmacological treatment of nearly all brain disorders[7,8], and extensive efforts are underway to discover targets on the human BBB to enhance drug delivery.

Specialized brain vascular properties arise from a complex community of interacting cell types[9–11]: endothelial cells, adjacent mural smooth muscle cells and pericytes, perivascular immune cells, and surrounding astrocytes that differ across brain regions and vary along an arteriovenous gradient[12]. Heterogeneity along this gradient produces functionally segmented circulatory, metabolic, and permeability properties necessary for brain health[3,5]. Pioneering work has profiled the mouse brain vasculature[12–15], but it remains unclear how conserved these findings are in humans. Single-nucleus studies have elucidated the cellular heterogeneity of the human brain in health and disease[16–19]. Yet, though vascular cell density[20,21] (70,000 cells/mm^3) approaches total glia density[20], prior studies, to our knowledge, have depleted human brain vas-

cular cells for unknown reasons. While human brain microvessels can be isolated for bulk assays, no method exists to extract nuclei from microvessels for analysis at single-cell resolution.

Given the scientific, medical, and pharmacological importance of the human brain vasculature, we set out to create a first single-cell atlas of the human brain vasculature of the hippocampus and cortex from control no cognitive impairment (NCI) and Alzheimer's disease (AD) individuals.

Cells of the human brain vasculature

Extracting nuclei from brain microvessels is a challenge because the method must dissociate the vessel basement membrane to release nuclei without damaging the nuclei themselves. We systematically explored enzymatic, chemical, and physical approaches. Most resulted in damaged nuclei devoid of RNA reads. We found success adapting a gentle protocol for splenocyte isolation – and combined it with extensive sucrose- and FACS-based cleanup to ensure high-quality data (Fig. 1a). We used our new method, VINE-seq (Vessel Isolation and Nuclei Extraction for Sequencing), to process 25 samples: the hippocampus of 9 AD and 8 age- and sex-matched NCI individuals, as well as the superior frontal cortex from a subset of 8 individuals (4 per group). Samples included a range of *APOE* genotypes, and NCI samples display no significant vascular pathologies in the studied hippocampus and cortex tissue. After quality-control, we obtained 143,793 single-nucleus transcriptomes. Visualization in uniform manifold approximation and projection (UMAP) space separated nuclei into 15 major cell types (Fig. 1b), including all known vascular and perivascular cell types: endothelial cells (arterial, capillary, venous), smooth muscle cells, pericytes, astrocytes, macrophages, T cells, and both perivascular and meningeal fibroblasts. The number of cerebrovascular nuclei captured here exceed those in the literature by several hundred-fold. Vascular cell type markers were validated in situ (Fig. 1c). Data can be browsed at https://twc-stanford.shinyapps.io/human_bbb.

VINE-seq captured three human brain immune cell types: microglia, macrophages, and T cells. Over 300 genes differentiated brain border macrophages and microglia, yielding new markers for human studies. Analysis of canonical markers indicated our capture of brain memory CD8 cytotoxic and CD4 T cells. Brain regional transcriptome differences were evident. Astrocyte transcriptional identity was the most influenced by brain region. Moreover, hippocampal endothelial cells demonstrated greater baseline inflammation, such as IFN-γ signaling, than those in

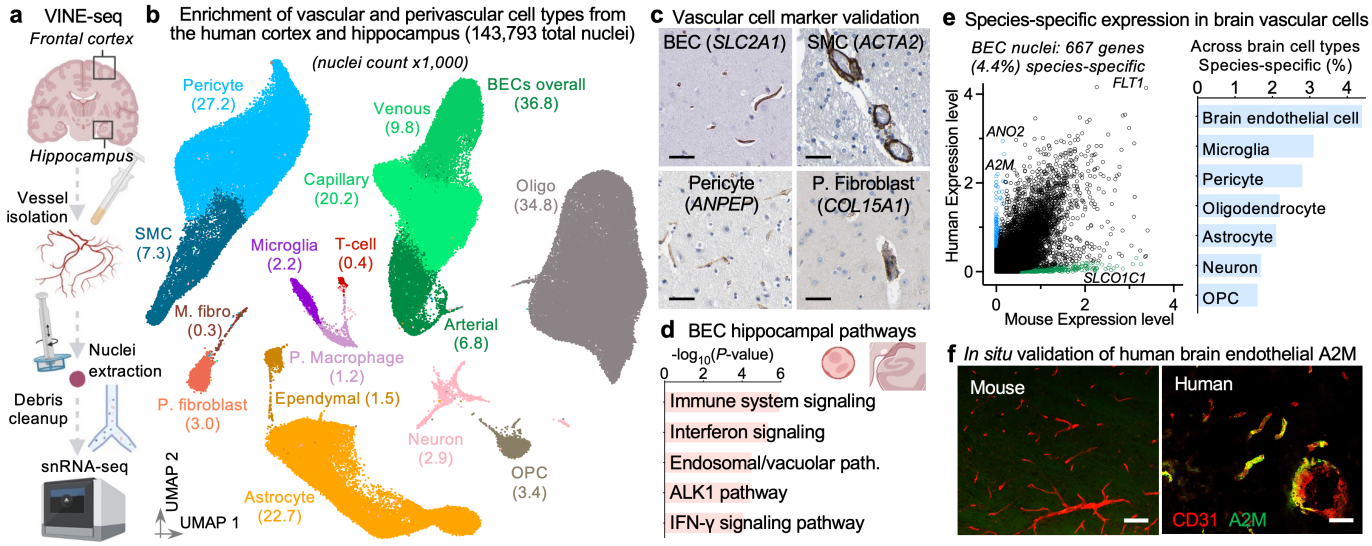

Figure 1. Cells of the human brain vasculature.

a, VINE-seq method to enrich vascular nuclei from postmortem human brain samples.

b, Uniform Manifold Approximation and Projection (UMAP) of 143,793 nuclei from 25 human hippocampus and superior frontal cortex samples across 17 individuals, colored by cell type and labeled with nuclei numbers.

c, Immunohistochemical validation of cell type-specific gene markers. Scale bar = 50 microns. Images from the Human Protein Atlas (http://www.proteinatlas.org)[25].

d, Enriched biological pathways in BECs from the hippocampus compared to the superior frontal cortex, in not cognitive impairment (NCI) individuals (*P* value < 0.05, cumulative hypergeometric test).

e, Scatter plot (left) depicting mRNA expression levels (logCPM) of mouse and human genes with one-to-one orthologs in BECs. Divergently expressed genes are colored (>10-fold difference, minimum 0.5 $\log_2$CPM expression). Proportion of each brain cell type's transcriptome that is specific to human versus mouse (same thresholds, right)

f, Immunohistochemical validation of A2M protein specifically in the human but not mouse vasculature. Scale bar = 50 microns.

cortex (Fig. 1d). Such inflammatory signaling may inhibit hippocampal neurogenesis, and together with accelerated hippocampal pericyte loss, provides a molecular hypothesis for hippocampal vasculature vulnerability and dysfunction[22].

We next compared nuclei transcriptomes between human and mouse endothelial cells and pericytes. Using a strict cutoff (>10x difference, logCPM>0.5), we found hundreds of species-enriched genes in brain endothelial cells (BECs) and pericytes (Fig. 1e). Understanding species differences in neurons and glia has been subject to intense study. Together with microglia[23], we find that BECs and pericytes exhibit the greatest transcriptional divergence (Fig. 1e). Several vascular solute transporters (e.g., GABA transporter *SLC6A12*) vary by species, suggesting differences in brain metabolism. Several genes of disease and pharmacological importance also vary. Together, the VINE-seq method opens the human brain vasculature for molecular study and provides an important data resource for interrogating its diverse cell types.

Organizing principles of human brain endothelial and mural cells

With our large-scale capture of vascular nuclei (>30x mouse[12], >200x human[16,17] prior studies), we sought to comprehensively characterize the molecular basis of endothelial and mural cell organization along the arteriovenous axis of the human brain vasculature. Changes along this axis are referred to as zonation[12,15]. Beginning with BECs, we clustered 36,825 nuclei into known arterial, capillary, and venous segments (Fig. 2a). Clusters were defined by established zonation markers, such as arterial *VEGFC* and *ALPL*; capillary *MFSD2A* and *SLC7A5*; and venous *IL1R1* and *NR2F2*[12,15]. While capillaries represent most (~90%) of the endothelium, VINE-seq captured rarer arterial (at 19%) and venous (27%) BECs, because of better strainer retention or nuclei liberation. We noticed a small cluster (571 nuclei, ~0.1%) outside conventional zonation. This cluster expressed genes characteristic of 'tip' cells (e.g., *PLAUR* and *LAMB1*) as well as 'proteostatic' heat shock proteins.

We next ordered endothelial nuclei along a one-dimensional pseudotime[24] axis to recapitulate the anatomical arteriovenous axis. Arterial and venous markers peaked at opposite ends, with capillary markers in between (Fig. 2b). We used the 665 most significantly variable cluster genes to unbiasedly order endothelial nuclei and observed seven gradually changing gene expression patterns, representing arterial, capillary, or venous markers, and combinations thereof (Fig. 2c). We confirmed that the Monocle range represented a cell order matching anatomical arteriovenous zonation via immunostaining[25]. Our patterns recapitulate the gradual/seamless zonation continuum described in mice – but interestingly, this similar overall continuum arises from significantly different component zonation markers.

We thus wondered whether established zonation markers in mice would be conserved in humans. We calculated a score measuring each gene's specificity to a given zonation (e.g., arterial, capillary, venous). Indeed, we observed in each vessel segment a significant number of markers that lost their zonation specificity between species (Fig. 2d). For example, the blood clotting gene von Willebrand factor (*VWF*) is largely expressed in mouse venous endothelial cells[12,15]. However, VWF is expressed throughout the human cerebrovasculature, even in small diameter capillaries (Fig. 2d-e). VWF abundance predisposes for ischemic stroke[26], raising implications for translational studies of stroke.

Next with mural cells, we clustered 34,508 nuclei into arterial smooth muscle cells (aSMCs), arteriolar SMCs (aaSMCs), and interestingly, two subclusters of pericytes (Fig. 3a-b). One pericyte subcluster enriched for small molecule transmembrane transport, which we call T-pericytes (for transport); while the

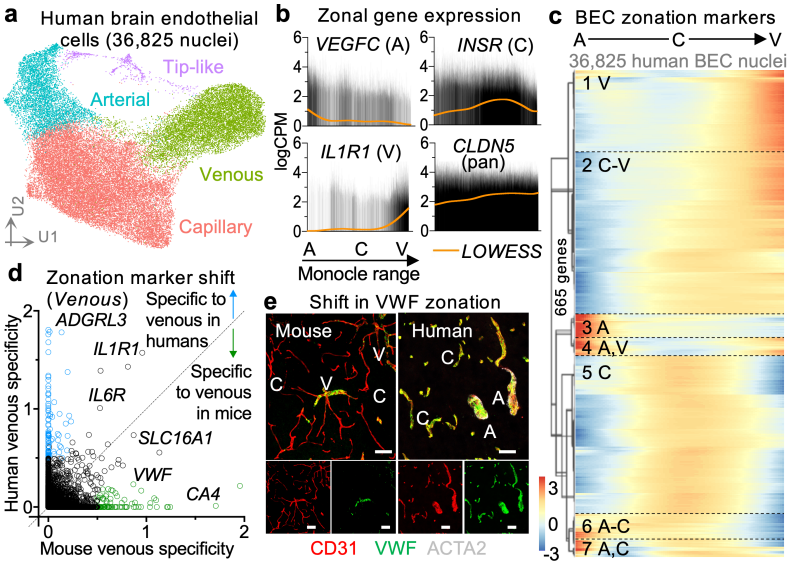

Figure 2. Organizing principles of human brain endothelial cells.

a, UMAP of 36,825 human brain endothelial cell (BEC) nuclei, colored by zonation.

b, Zonal expression of transcripts across human BECs ordered by Monocle pseudotime. LOWESS regression line (orange) and density of black lines (counts) correspond with expression levels. A = arterial, C = capillary, and V = venous.

c, Heatmap of zonation-dependent gene expression in human BECs.

d, Scatter plot depicting the specificity of transcripts for venous BECs in mice[12] versus humans. Venous specificity score = avg(logFC(vein/cap), logFC(vein/art)). For example, *VWF* is predicted to be more specific to venous BECs in mice than it is in humans.

e, Immunohistochemical validation of VWF specificity to venous BECs in mice but not in humans. Scale bar = 50 microns.

other for extracellular matrix (ECM) organization, M-pericytes (for matrix). Thus, function rather than anatomical location may be the major driver of human pericyte transcriptional identity. M-pericytes may thus contribute to small vessel diseases like CADASIL, CARASIL, and Collagen IV deficiencies for which perturbations in the cerebrovascular ECM cause disease[27]. Moreover, human T-pericytes – but not mouse pericytes – express transporters like the GABA transporter *SLC6A1* (involved in epilepsy) and glutamate transporter *SLC1A3*. Because recent mouse pericyte datasets have cautioned against endothelial contamination, we assessed and found no such contamination in our human pericyte nuclei.

To study mural cell zonation, we used the 670 most significantly variable and robustly expressed cluster genes to order all mural cell nuclei and observed the expected order of aSMC markers on one end (e.g., *ACTA2, TAGLN*), followed by aaSMC (e.g., *SLIT3, CTNNA3*); and pericyte markers on the other end (e.g., *ABCC9, PTN*), confirmed *in situ* (Fig. 3c). Recapitulating patterns described in mice[12] – and as opposed to the gradual zonation pattern in BECs – , human mural cells exhibited an abrupt gene expression transition between SMCs and pericytes (Fig. 3c). To determine whether T- and M-pericyte ordering (Fig. 3c) reflects anatomical arteriovenous order, we mapped them to mouse mural cells markers*12* and found no differences in their expression of mouse capillary 'Pericyte' versus venous mural cell 'vSMC' markers (Fig. 3d). This suggests that T- and M-pericytes do not segregate by arteriovenous segment. Immunostaining confirmed that T- and M-pericyte markers are each present across both small and large diameter brain vessels.

Together, these data suggest that human pericytes transcriptionally identify more by functional specialization than by arteriovenous location; and that while aSMCs and aaSMCs reside on arterial and arteriolar vessels respectively, T- and M-pericytes intermix along the capillary and venous vasculature. As with BECs, we find that only a minority of top mouse SMC and pericyte markers retain their predictive value in humans (Fig 3e). Because proteins encoded by zonation marker genes perform a variety of important functions at defined arteriovenous locations, species-specific endothelial and mural cell differences likely reflect fundamental differences in brain vascular properties that can now be studied.

Molecular definitions for perivascular and meningeal fibroblasts

Using annotations from recent mouse studies[12,28], we noticed fibroblasts from both the human brain vascular and meningeal barriers (Fig. 4a). Cooperating with the vascular BBB, the recently (re)discovered meningeal lymphatics plays important roles in waste clearance and neuroimmune surveillance[29,30]. Fibroblasts transcriptionally segregated according to anatomical location: vascular versus meningeal (Fig. 4a-b), but also separated according to meningeal layers. Pathway enrichment analysis of marker genes revealed specialized fibroblast functions (Fig. 4b): perivascular fibroblasts showed enriched expression for ECM structural components or its modifiers/receptors (e.g., "TGF-β regulation of ECM"), while meningeal fibroblasts enriched for solute transporters. Fibrotic scars arise from the pathological deposition of Collagen I (COL1)-rich ECM. Consistent with elegant mouse lineage tracing studies[31], our work indicates that human perivascular fibroblasts are the main producers of COL1 in the brain and thus likely form fibrotic scars after brain injury.

Closer comparison of differentially expressed genes (DEGs) be-

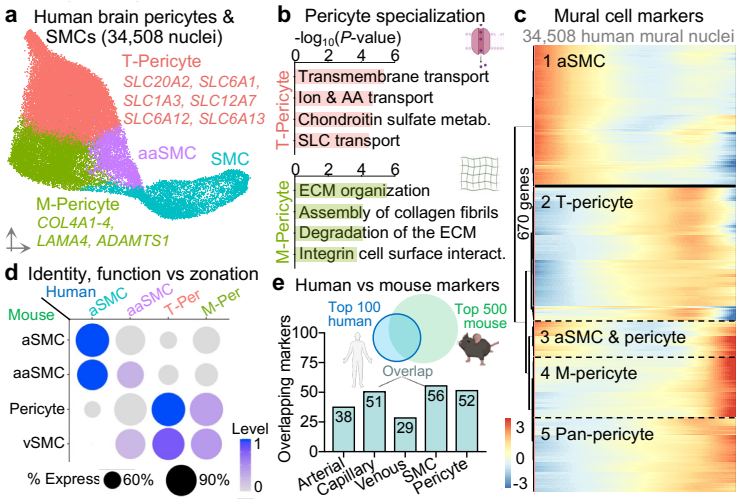

Figure 3. Organizing principles of human brain mural cells.

a, UMAP of 34,508 human pericyte and smooth muscle cell nuclei, colored by cell subtype. aSMC = arterial smooth muscle cell (aSMC), aaSMC = arteriole SMCs, T-Pericyte = solute transport pericytes, and M-Pericyte = Extracellular matrix regulating pericytes.

b, Enriched biological pathways in T- and M-pericytes compared to remaining SMC and pericyte populations (P value < 0.05, cumulative hypergeometric test).

c, Heatmap of gene expression in human SMCs and pericytes. Solid line delineating aaSMC/aSMCs from pericytes reflects an abrupt transcriptomic transition. Note that unlike BECs, mural cell ordering does not reflect anatomical arteriovenous ordering.

c, Mapping expression of mouse mural cell markers onto human mural cell types. The top 500 mouse markers were aggregated into four distinct modules ('aSMCs', 'aaSMCs', capillary 'pericytes', venous smooth muscle cells 'vSMCs')[12] and their expression assessed in the four transcriptionally distinct human mural cell types.

i, Overlap between the top 100 human endothelial and mural cell subtype markers and those identified in mice. A more lenient set of 500 mouse markers[12] was used for comparison for robust results. Note that species-conservation of a cell type marker depends on species-specific changes in the given cell type and changes among remaining cell types.

tween fibroblast populations revealed a polarization of solute influx and efflux pumps: meningeal fibroblasts specifically expressed SLC influx solute transporters, while perivascular fibroblasts exclusively expressed ABC efflux pumps (Fig. 4d). We confirmed this polarized expression *in situ* (Fig. 4e). Perivascular fibroblasts reside in the Virchow-Robin space, and thus like meningeal fibroblasts, bathe in the cerebrospinal fluid (CSF). This cooperative circuit of polarized transporters suggests fibroblast regulation of solute exchange between the brain and CSF[32]. Finally, as in endothelial and mural cells, perivascular fibroblast-like cell markers varied by species. Together, these data provide a first characterization of human brain fibroblast diversity, revealing the molecular basis of their anatomical specialization and a cooperative circuit for CSF solute exchange.

Vascular cell-type specific perturbations in Alzheimer's disease

AD is a neurodegenerative disorder with progressive impairment of cognitive function leading to dementia. Impairment arises from complex perturbations in cell composition and gene expression. We thus sought to profile changes in the AD human brain vasculature at single-cell resolution. We defined our patient groups by clinical diagnosis and confirmed the presence of β-amyloid plaques in the hippocampus and cortex.

Recent studies have identified context-dependent, disease associated glial subpopulations[16,17,33]. We did not observe new vascular cell subclusters emerge in AD (Fig. 5a). In contrast, we found a strong loss of brain vascular nuclei—across endothelial, SMC, pericyte, and fibroblast-like cells (Fig. 5a-b). Thus, beyond focal cerebrovascular damage, vascular loss may be widespread across the arteriovenous axis. Among pericytes, M-pericytes involved in ECM organization exhibited selective vulnerability (Fig. 5b), providing a molecular hypothesis for structural BBB breakdown reported in AD[22]. Yet, FACS and snRNA-seq approaches may not always yield reliable cell quantifications. Thus, we stained hippocampal tissue to quantify the number of Hoechst+ vascular cells within Collagen IV⁺ cerebrovasculature. While we found no significant differences in the area of Collagen IV⁺ vessels or total number of Hoechst⁺ nuclei, we found a significant decrease with AD in the number of Hoechst+ nuclei within Collagen IV⁺ vessels, consistent with reports of "string vessels" in disease[34] (Fig. 5b). In short, we find selective vulnerability of specific vascular cell subpopulations in AD.

We next examined cell type-specific gene expression changes in AD. We identified 463 unique differentially expressed genes using more stringent thresholds (DEGs,

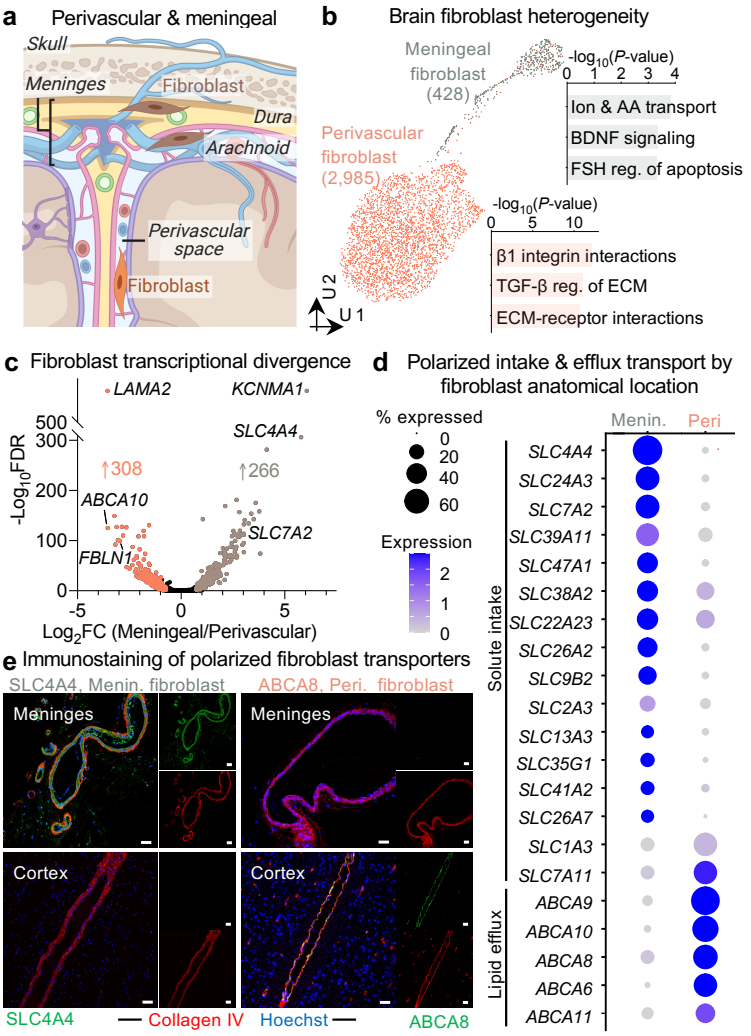

Figure 4. Molecular definitions for brain perivascular and meningeal fibroblasts.

a, Anatomical reference of the human meninges (dura and arachnoid) and perivascular space, each with a resident fibroblast population.

b, UMAP of 2,985 human perivascular fibroblast-like nuclei and 428 meningeal fibroblast nuclei. Enriched biological pathways derived from respective fibroblast cell type markers (*P* value < 0.05, cumulative hypergeometric test).

c, Differentially expressed genes between perivascular (left, orange) and meningeal (right, brown) fibroblasts (MAST, Benjamini Hochberg correction; FDR < 0.01 and logFC>0.5 [log_2FC>0.72] to be colored significant).

d, Expression of all differentially expressed (from (c)) SLC and ABC family members across perivascular and meningeal fibroblasts.

e, Immunostaining validation of polarized meningeal and perivascular fibroblast transporter expression: the meningeal fibroblast-specific influx pump SLC4A4 (left, green) stains selectively in the meninges but not cortical vasculature (red); and vice versa for the perivascular-specific efflux pump ABCA8 (right, green). Scale = 50 μM.

Fig. 5c). Overall, mural cells exhibited the strongest changes, and other cell types exhibited a signature of gene repression: 61-78% of DEGs were downregulated (Fig. 5c). DEGs were robustly detected across different levels of expression. Most DEGs were cell type- and zonation-specific, suggesting a heterogenous response to AD pathology across the vasculature. Intriguingly, several DEGs are risk genes implicated in AD and small vessel disease GWAS studies. At the pathway level, DEGs in mural cells and fibroblasts implicated dysregulated vasoconstriction and compromised blood flow (Fig. 5d). This provides a potential molecular mechanism for cerebral hypoperfusion discernible in MRI of living AD patients[35].

APOE4 carriers may exhibit accelerated BBB breakdown before cognitive impairment[36]. We found dramatic interferon inflammation in the endothelium of *APOE4* carriers (Fig. 5e). Next, we compared AD DEGs in BECs with those in mouse models of AD. Such models have facilitated mechanistic study of β-amyloid pathology, but recent reports describe significant species differences in various cell types, like microglia[23]. We isolated BECs from 12-14 month old Thy1-hAPP^{Lon,Swe} mice[37] (and littermate wild-type controls) and processed them for single-cell sequencing. Surprisingly, we observed minimal overlap between human AD and mouse hAPP BEC DEGs (Fig. 5f). Finally, because AD pathology begins and spreads via a strikingly consistent regional pattern, we assessed the impact of AD on brain regional vascular specialization. We found regional differences in cell density and transcriptional profiles largely erased in AD – suggesting impairments in brain region-specific vascular functions.

Together, these findings show that AD patients exhibit heterogeneous cell type-, zonation-, region-, and species-specific vulnerabilities and perturbations across the brain vasculature that require single-cell approaches to profile.

AD GWAS disease variants enriched in the human brain vasculature

A major goal of biomedical research is to understand how genetic variation contributes to disease. GWAS studies have nominated genes contributing to AD risk, though their cell type contexts were unknown. Recent studies strongly implicate microglia as the major AD GWAS-expressing cell type[23,38–41]. We wondered, however, whether the unintended depletion of brain vascular cells prematurely dismissed their potential contributions. We curated AD GWAS studies[39–41] to order the top 45 risk genes. With our expanded survey of brain cell types, we calculated the cell type proportional expression for each GWAS gene using Expression Weighted Cell Type Enrichment (EWCE)[42]. We indeed observed among brain parenchymal cells a specific microglial signature for top AD GWAS genes such as *TREM2, MS4A6A, CR1*, and *SPI1* that are now under intense mechanistic study (Fig. 6a, right).

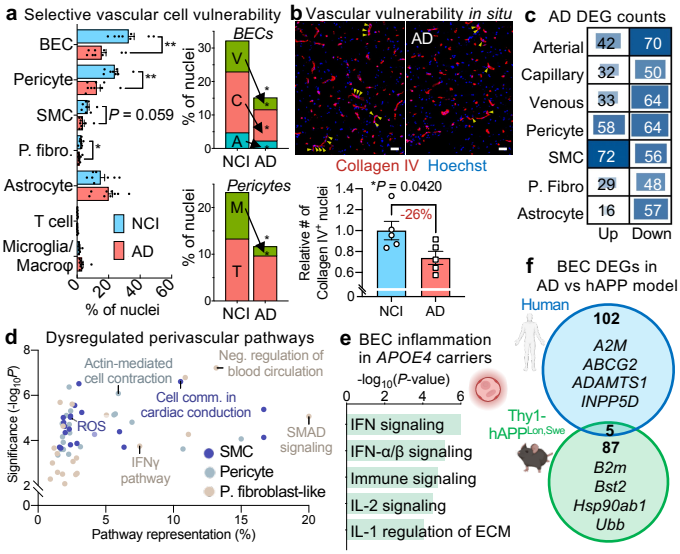

Figure 5. Vascular cell-type specific perturbations in Alzheimer's disease.

a, Proportion of cell types captured in Alzheimer's disease (AD) and no cognitive impairment individuals (NCI) (left). Proportion of BEC and pericyte subpopulations in AD and NCI (right) (n = 8 NCI, n = 9 AD, two-sided t-test; mean +/- s.e.m.). **BEC P = 0.002, **Pericyte P = 0.003, *P. fibroblast P = 0.0461.

b, Immunohistochemical validation of a loss of vascular cell density in AD (number of Hoechst$^+$ nuclei within Collagen IV$^+$ vasculature). Scale bar = 50 microns (n = 5 NCI and AD, nested two-sided t-test; mean +/- s.e.m.). Yellow arrows denote example vascular nuclei.

c, Differentially expressed gene (DEG) counts for each cell type in AD. The intensity of the blue color and the size of the squares are proportional to entry values.

d, Enriched biological pathways from AD differentially expressed genes in pericytes, smooth muscle cells, and perivascular fibroblast-like cells, plotted by Pathway Representation (in a given pathway, what proportion of all members are DEGs) and Significance (-log$_{10}$P) of pathway enrichment.

e, Enriched biological pathways from genes upregulated in AD *APOE4* carriers in capillary and venous endothelial cells (P value < 0.05, cumulative hypergeometric test).

f, Venn diagram comparing DEG BECs in human AD samples compared to those from the Thy1-hAPP T41B^{Lon,Swe} amyloidosis mouse model[37]. Note that only genes with human-mouse orthologs are shown, and that the absolute logFC threshold for calling DEGs in mouse APP BECs was lowered to 0.15 (by half) to ensure claims of limited overlap with human BECs were robust.

Intriguingly, we noticed several GWAS genes were strongly expressed in human brain vascular and perivascular cell types (Fig. 6a, left). This included two genes previously implicated in the mouse vasculature, *PICALM* and *CD2AP*[43,44]. But this also included surprising genes, such as the immune-related *PLCG2* and *HLA-DRB1/5* in arterial cells, endocytic *INPP5D* and *USP6NL* in capillaries, and ECM-related ADAMTS1, *ADAMTS4*, *FERMT2*, and *AGRN* in SMCs and pericytes (Fig. 6a). Within pericytes, expression varied

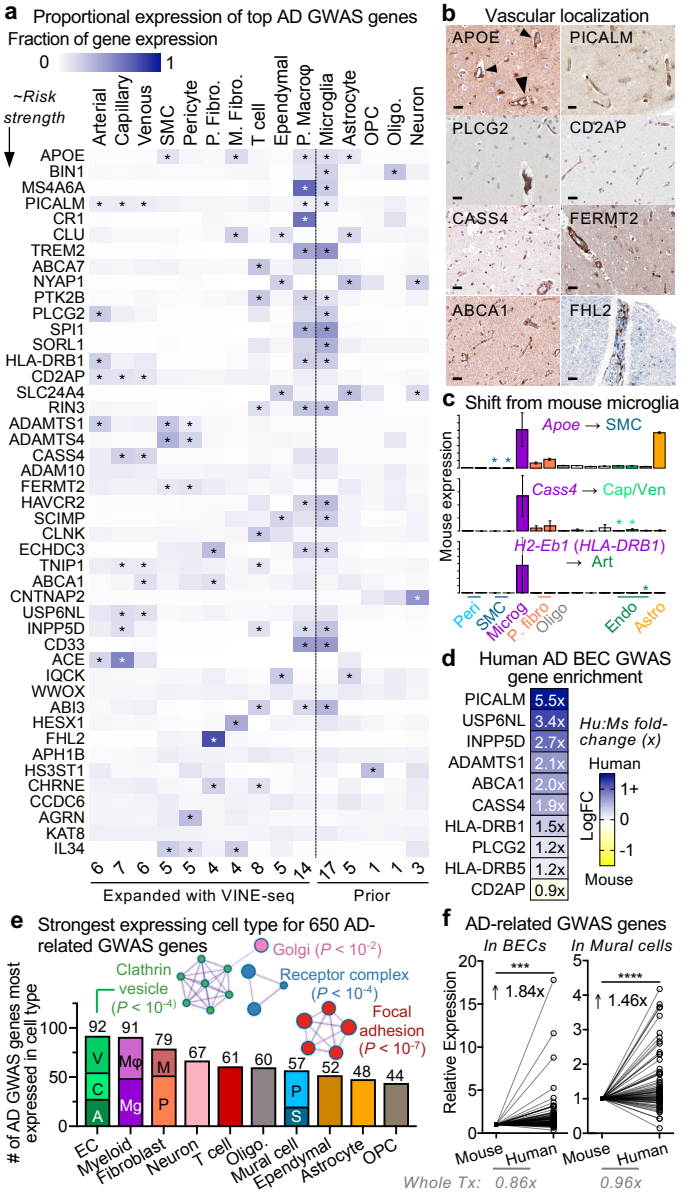

Figure 6. GWAS disease variants are enriched in the human brain vasculature.

a, Proportional expression of the top 45 AD GWAS genes across all major brain cell types. Expression values for a given gene sums to 1 across cell types using the EWCE method[42]. Genes ordered in approximate risk strength[39–41,51]. Asterisks denote strongest expressing cell types. Cells to the left of dashed line are from the vasculature, newly added here; to the right, parenchymal cells captured before. Numbers on the bottom summarize the number of GWAS genes enriched in a given cell type.

Note: MSA46A represents the average expression of MS4A46A, *MS4A4A*, and *MS4A4E*; likewise, *HLA-DRB1* averages *HLA-DRB1* and *HLA-DRB5*. EPHA1 was not robustly detected.

b, Immunohistochemical confirmation of vascular localization of proteins encoded by top AD GWAS genes from (**a**). Scale bar = 25 microns. Arrowheads in APOE point to signal around larger diameter vessels, consistent with SMC expression. Images from the Human Protein Atlas (*http://www.proteinatlas.org*)[25].

c, Examples of genes expressed specifically in mouse microglia but then also expressed in human brain vascular cell types (*n* of ~3,500 whole cell mouse transcriptomes[12], mean value +/- SEM).

d, BEC heatmap of top AD GWAS genes colored by logFC(human/mouse) and labeled by the linear fold-change (human/mouse) value.

e, Quantification of the number of AD and AD-related trait GWAS genes[17] most expressed in a given cell type. 383 of 651 genes (59%) mapped to vascular or perivascular cell types. PPI network of GO Cellular Components (*P* value < 0.05, cumulative hypergeometric test).

A = arterial, C = capillary, V = venous endothelial cell (EC). Mg = microglia, and Mφ = macrophage. In fibroblasts, M = meningeal and P = perivascular. In mural cells, S = SMC and P = pericyte.

f, Human enrichment of AD-related trait GWAS genes[17] highest expressed in BECs (left) and mural cells (right). In contrast to GWAS genes, the ratio of human to mouse expression across the overall transcriptome is less than or ~1 for both cell types (bottom, paired two-sided t-test, $^{****}P < 0.0001$ and $^{***}P = 0.0002$).

across M- and T-pericyte subtypes. *APOE*, linked to myeloid cells and astrocytes, was robustly expressed in human SMCs and meningeal fibroblasts. Several GWAS genes like *ABCA7* and *CLNK* enrich in T cells, and independent datasets show minimal expression in other brain cell types. Several genes like *ABCA1*, *FHL2*, *HESX1*, and *IL34* enrich in fibroblasts. Importantly, we confirmed our findings via immunostaining of proteins encoded by vascular GWAS genes, such as CASS4, FERMT2, PLCG2, and FHL2 (Fig. 6b). Most GWAS genes were expressed similarly between the hippocampus and cortex. In total, at least 30 of the top 45 AD GWAS genes are enriched in cells of the human brain vasculature (not including those solely in perivascular macrophages), suggesting thorough vascular and perivascular involvement in AD.

We next wondered whether these genes are expressed in different cell types between mice and humans. Many genes like *APOE*, *CASS4*, *INPP5D*, and *HLA-DRB1* were predominately microglial in mice[12] but then also exhibited vascular expression in humans (Fig. 6c). Nearly every top AD GWAS gene expressed in BECs showed greater expression in humans (Fig. 6d). Together, these data suggest a partial evolutionary transfer of AD risk genes and pathways from microglia to the vasculature from mice to humans.

We broadened our scope to hundreds of GWAS genes for AD and AD-related traits[17]. We observed robust, cell type-specific vascular and perivascular expression. For each gene, we assigned the cell type with the strongest expression, discovering surprisingly, that BECs harbored the most AD-related GWAS genes, followed by microglia/ macrophages (Fig. 6e). Within BECs, AD-related GWAS

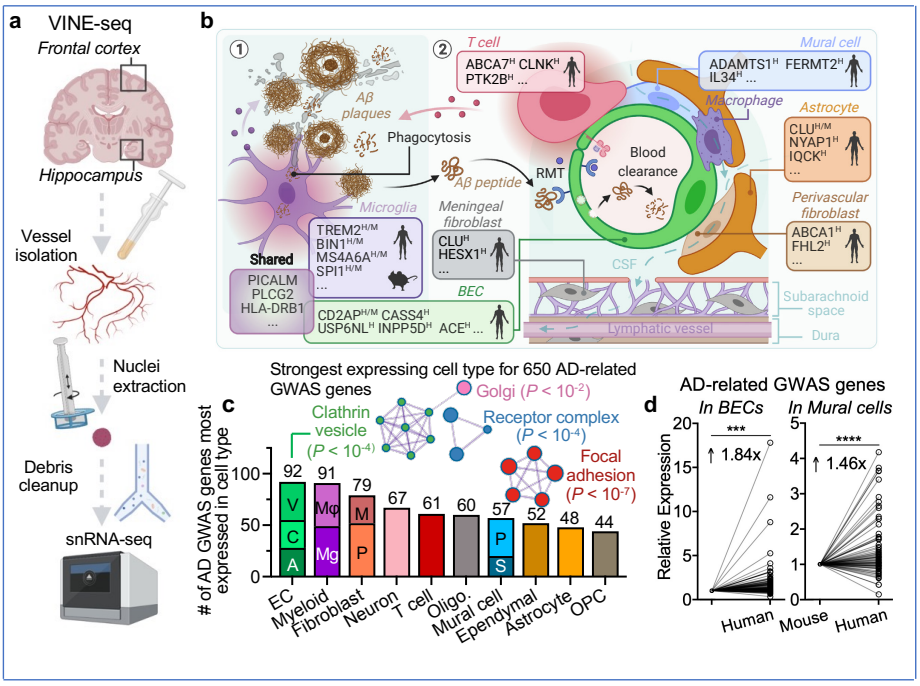

Figure 7. A model for how AD risk genes may dysregulate homeostatic functions maintained by human brain vascular cell types.

Summary of the GWAS-prioritized genes associated with Alzheimer's disease (AD) that are enriched in human brain vascular cell types. Many of these cells and genes are involved in waste clearance (including Aβ plaques and peptides) from the brain, or in neuroinflammation. Gene expression in humans (H) and/or mice (M) is indicated. While microglia are still frontline participants ("1"), the dramatic expansion of the human brain and metabolism may necessitate enhanced waste clearance and neuroimmune surveillance from vascular cell types ("2") to support brain homeostasis.

genes enriched in protein endo- and transcytosis components, such as receptor and clathrin vesicle components (Fig. 6d-e). We recently demonstrated a decline in BEC clathrin-mediated transcytosis8 with age, suggesting one mechanism by which aging and risk genes converge to increase AD risk. In total, over half of AD-related GWAS genes mapped to vascular or perivascular cell types (383 of 651). As with top GWAS genes, we observed enhanced human over mouse expression of AD-related genes in BECs and pericytes (Fig. 6f). Importantly, this human-enhanced expression is not observed for the whole transcriptome.

Together, these data provide a more comprehensive understanding of the diverse cell types contributing to AD risk. We suggest that an evolutionarily expanded vascular-microglia axis underlies the genetic risk for AD via shared protein clearance (BEC-microglia) and inflammatory pathways (BEC-T cell-microglia) (Fig. 6e, Fig. 7).

Discussion

We report here 143,793 single-cell, genome-wide quantitative transcriptomes from the human brain vasculature in health and AD. We molecularly define the principal vascular cell types; their differences by brain region and species; and the organizational principles of endothelial, mural, and fibroblast-like cells. We reveal selective vulnerability of vascular subpopulations and transcriptomic perturbations associated with clinically-diagnosed AD; and the unexpected expression of AD GWAS genes across human brain vascular cell types, confirmed in situ. Data are available to browse at https://twc-stanford.shinyapps.io/human_bbb.

How do human vascular GWAS genes fit into established AD pathways? Current understanding implicates β-amyloid metabolism, cholesterol/lipid dysfunction, innate immunity, and endocytosis[43]. We confirm these pathways and expand the cell types involved, for example, debris clearance via BEC clathrin-mediated endocytosis – and adaptive T cell in addition to innate immunity. We propose that the expansion of the human brain, brain activity, and activity byproducts (like β-amyloid[45]) necessitate enhanced clearance mechanisms and neuroimmune surveillance. In this model, microglia are still frontline participants in AD pathogenesis. But more than in mice, human vascular and perivascular cells partake. For example, microglial clearance functions can become overwhelmed46, diverting debris to BECs. Indeed, microglial depletion results in cerebral amyloid angiopathy[47]. But unlike microglia, vascular cells do not proliferate efficiently. Thus, constant vascular exposure to debris like β-amyloid triggers cell death, dysfunction, and impaired blood and CSF flow. Recent work identified a CD8 T_{EMRA} population clonally expanded in AD CSF[48], potentially influenced by T cell GWAS genes. Together, we suggest an intertwined microglia-vascular axis expanded in humans, with vascular cells playing an auxiliary role via shared endocytosis and inflammatory pathways. We note though the likelihood of additional human brain vascular contributions, as evidenced by SMC, pericyte, and fibroblast-enriched GWAS genes of unclear function. In general, disease risk variants enrich in gene expression-regulating enhancer regions[49] that undergo accelerated species divergence[50], providing one potential explanation for human-specific expression and dysregulation of AD risk genes in cerebrovascular cell types.

The field now has a near complete census of human brain cell types. This atlas expands by orders of magnitude the number of human brain vascular cell type and zonation markers to inform research, such as identifying vessels, validating organoid fidelity, or deconvoluting bulk RNA-seq datasets. Our work also facilitates translational opportunities. This dataset provides targets

for antibodies and other modalities to deliver therapeutics to the brain. VINE-seq enables study of brain vascular contributions to various diseases, such as stroke, multiple sclerosis, and COVID-19. But as with recent snRNA-seq studies[16–19], it will be important to distinguish which vascular transcriptional perturbations respond to versus drive disease, clarify their links to clinical and pathologic traits, and dissect the mechanisms by which vascular-expressed AD variants confer disease risk. Overall, VINE-seq and the ensuing single-cell atlas provide a blueprint for studying the molecular basis of the human brain vasculature.

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I thank our funding agencies for supporting our research, including the National Institute on Aging, the Simons Foundation, and the AHA-Allen Initiative in Brain Health and Cognitive Impairment. Finally, I thank the Werner Risau Prize committee and the DGZ for recognizing this work with the Werner Risau Prize. I am deeply honored and thankful to be part of the vascular biology community, and I look forward to the exciting conversations and collaborations in the years to come.

References

1. Feigin, V. L. *et al.* Global and regional burden of stroke during 1990-2010: Findings from the Global Burden of Disease Study 2010. *Lancet* (2014). doi:10.1016/S0140-6736(13)61953-4
2. Chow, B. W. & Gu, C. The Molecular Constituents of the Blood-Brain Barrier. *Trends Neurosci.* **38**, 598–608 (2015).
3. Profaci, C. P., Munji, R. N., Pulido, R. S. & Daneman, R. The blood-brain barrier in health and disease: Important unanswered questions. *J. Exp. Med.* (2020). doi:10.1084/jem.20190062
4. Obermeier, B., Daneman, R. & Ransohoff, R. M. Development, maintenance and disruption of the blood-brain barrier. *Nature Medicine* (2013). doi:10.1038/nm.3407
5. Sweeney, M. D., Zhao, Z., Montagne, A., Nelson, A. R. & Zlokovic, B. V. Blood-brain barrier: From physiology to disease and back. *Physiological Reviews* (2019). doi:10.1152/physrev.00050.2017
6. Iadecola, C. The Pathobiology of Vascular Dementia. *Neuron* (2013). doi:10.1016/j.neuron.2013.10.008
7. Pardridge, W. M. Drug transport across the blood-brain barrier. *Journal of Cerebral Blood Flow and Metabolism* (2012). doi:10.1038/jcbfm.2012.126
8. Yang, A. C. *et al.* Physiological blood–brain transport is impaired with age by a shift in transcytosis. *Nature* (2020). doi:10.1038/s41586-020-2453-z
9. Daneman, R., Zhou, L., Kebede, A. A. & Barres, B. A. Pericytes are required for blood–brain barrier integrity during embryogenesis. *Nature* **468**, 562–566 (2010).
10. Armulik, A. *et al.* Pericytes regulate the blood-brain barrier. *Nature* **468**, 557–561 (2010).
11. Janzer, R. C. & Raff, M. C. Astrocytes induce blood-brain barrier properties in endothelial cells. *Nature* (1987). doi:10.1038/325253a0
12. Vanlandewijck, M. *et al.* A molecular atlas of cell types and zonation in the brain vasculature. *Nature* **554**, 475–480 (2018).
13. Sabbagh, M. F. *et al.* Transcriptional and epigenomic landscapes of CNS and non-CNS vascular endothelial cells. *Elife* (2018). doi:10.7554/elife.36187
14. Kalucka, J. *et al.* Single-Cell Transcriptome Atlas of Murine Endothelial Cells. *Cell* (2020). doi:10.1016/j.cell.2020.01.015
15. Chen, M. B. *et al.* Brain Endothelial Cells Are Exquisite Sensors of Age-Related Circulatory Cues. *Cell Rep.* (2020). doi:10.1016/j.celrep.2020.03.012
16. Mathys, H. *et al.* Single-cell transcriptomic analysis of Alzheimer's disease. *Nature* (2019). doi:10.1038/s41586-019-1195-2
17. Grubman, A. *et al.* A single-cell atlas of entorhinal cortex from individuals with Alzheimer's disease reveals cell-type-specific gene expression regulation. *Nat. Neurosci.* (2019). doi:10.1038/s41593-019-0539-4
18. Jäkel, S. *et al.* Altered human oligodendrocyte heterogeneity in multiple sclerosis. *Nature* (2019). doi:10.1038/s41586-019-0903-2
19. Velmeshev, D. *et al.* Single-cell genomics identifies cell type–specific molecular changes in autism. *Science (80-.).* (2019). doi:10.1126/science.aav8130
20. Keller, D., Erö, C. & Markram, H. Cell densities in the mouse brain: A systematic review. *Frontiers in Neuroanatomy* (2018). doi:10.3389/fnana.2018.00083
21. Niedowicz, D. M. *et al.* Obesity and diabetes cause cognitive dysfunction in the absence of accelerated β-amyloid deposition in a novel murine model of mixed or vascular dementia. *Acta Neuropathol. Commun.* (2014). doi:10.1186/2051-5960-2-64
22. Montagne, A. *et al.* Blood-Brain barrier breakdown in the aging human hippocampus. *Neuron* **85**, 296–302 (2015).
23. Geirsdottir, L. *et al.* Cross-Species Single-Cell Analysis Reveals Divergence of the Primate Microglia Program. *Cell* (2019). doi:10.1016/j.cell.2019.11.010
24. Trapnell, C. *et al.* The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. *Nat. Biotechnol.* (2014). doi:10.1038/nbt.2859
25. Uhlén, M. *et al.* Tissue-based map of the human proteome. *Science (80-.).* (2015). doi:10.1126/science.1260419
26. De Meyer, S. F., Stoll, G., Wagner, D. D. & Kleinschnitz, C. Von Willebrand factor: An emerging target in stroke therapy. *Stroke* (2012). doi:10.1161/STROKEAHA.111.628867
27. Mao, M., Alavi, M. V., Labelle-Dumais, C. & Gould, D. B. Type IV Collagens and Basement Membrane Diseases: Cell Biology and Pathogenic Mechanisms. *Curr. Top. Membr.* (2015). doi:10.1016/bs.ctm.2015.09.002
28. DeSisto, J. *et al.* Single-Cell Transcriptomic Analyses of the Developing Meninges Reveal Meningeal Fibroblast Diversity and Function. *Dev. Cell* (2020). doi:10.1016/j.devcel.2020.06.009
29. Louveau, A. *et al.* Structural and functional features of central nervous system lymphatic vessels. Nature (2015). doi:10.1038/nature14432
30. Aspelund, A. *et al.* A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules. *J. Exp. Med.* (2015). doi:10.1084/jem.20142290
31. Dorrier, C. E. *et al.* CNS fibroblasts form a fibrotic scar in response to immune cell infiltration. *Nat. Neurosci.* (2021). doi:10.1038/s41593-020-00770-9
32. Iliff, J. J. *et al.* A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid β. *Sci. Transl. Med.* (2012). doi:10.1126/scitranslmed.3003748
33. Keren-Shaul, H. *et al.* A Unique Microglia Type Associated with Restricting Development of Alzheimer's Disease. *Cell* (2017). doi:10.1016/j.cell.2017.05.018
34. Brown, W. R. A review of string vessels or collapsed, empty basement membrane tubes. *Journal of Alzheimer's Disease* (2010). doi:10.3233/JAD-2010-100219
35. Roher, A. E. *et al.* Cerebral blood flow in Alzheimer's disease. *Vasc. Health Risk Manag.* (2012). doi:10.2147/VHRM.S34874
36. Montagne, A. *et al.* APOE4 leads to blood–brain barrier dysfunction predicting cognitive decline. *Nature* (2020). doi:10.1038/s41586-020-2247-3
37. Rockenstein, E., Mallory, M., Mante, M., Sisk, A. & Masliaha, E. Early formation of mature amyloid-β protein deposits in a mutant APP transgenic model depends on levels of Aβ1-42. *J. Neurosci. Res.* (2001). doi:10.1002/jnr.1247
38. Nott, A. *et al.* Brain cell type–specific enhancer–promoter interactome maps and disease-risk association. *Science (80-.).* (2019). doi:10.1126/science.aay0793
39. Lambert, J. C. *et al.* Meta-analysis of 74,046 individuals identifies 11 new susceptibility loci for Alzheimer's disease. *Nat. Genet.* (2013). doi:10.1038/ng.2802

40. Kunkle, B. W. *et al.* Genetic meta-analysis of diagnosed Alzheimer's disease identifies new risk loci and implicates Aβ, tau, immunity and lipid processing. *Nat. Genet.* (2019). doi:10.1038/s41588-019-0358-2
41. Jansen, I. E. *et al.* Genome-wide meta-analysis identifies new loci and functional pathways influencing Alzheimer's disease risk. *Nat. Genet.* (2019). doi:10.1038/s41588-018-0311-9
42. Skene, N. G. & Grant, S. G. N. Identification of vulnerable cell types in major brain disorders using single cell transcriptomes and expression weighted cell type enrichment. *Front. Neurosci.* (2016). doi:10.3389/fnins.2016.00016
43. Karch, C. M. & Goate, A. M. Alzheimer's disease risk genes and mechanisms of disease pathogenesis. *Biological Psychiatry* (2015). doi:10.1016/j.biopsych.2014.05.006
44. Zhao, Z. *et al.* Central role for PICALM in amyloid-β blood-brain barrier transcytosis and clearance. *Nat. Neurosci.* (2015). doi:10.1038/nn.4025
45. Cirrito, J. R. *et al.* Synaptic activity regulates interstitial fluid amyloid-β levels in vivo. *Neuron* (2005). doi:10.1016/j.neuron.2005.10.028
46. Safaiyan, S. *et al.* Age-related myelin degradation burdens the clearance function of microglia during aging. *Nat. Neurosci.* (2016). doi:10.1038/nn.4325
47. Spangenberg, E. *et al.* Sustained microglial depletion with CSF1R inhibitor impairs parenchymal plaque development in an Alzheimer's disease model. *Nat. Commun.* (2019). doi:10.1038/s41467-019-11674-z
48. Gate, D. *et al.* Clonally expanded CD8 T cells patrol the cerebrospinal fluid in Alzheimer's disease. *Nature* (2020). doi:10.1038/s41586-019-1895-7
49. Farh, K. K. H. *et al.* Genetic and epigenetic fine mapping of causal autoimmune disease variants. *Nature* (2015). doi:10.1038/nature13835
50. Villar, D. *et al.* Enhancer evolution across 20 mammalian species. *Cell* (2015). doi:10.1016/j.cell.2015.01.006

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I'm an engineer-turned-neuroscientist developing new molecular tools to systematically study the meaning, mechanisms, and therapeutic potential of protein and immune crosstalk across the brain's unique barriers.

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MS in Bioengineering	9/2015-9/2017
Massachusetts Institute of Technology	Cambridge, MA
Majors: Mechanical Engineering, Materials Science and Engineering	9/2009-6/2013
Minors: Biomedical Engineering, Public Policy	

B. Publications

First Author (5)

1. **Yang AC**[‡], Vest RT[#], Kern F[#], Lee DP, Maat CA, Losada PM, Chen MB, Agam MR, Schaum N, Khoury N, Calcuttawala K, Pálovics R, Shin A, ... Siegenthaler JA, McNerney WM, Keller A, Wyss-Coray T[†].
   A human brain vascular atlas reveals diverse cell mediators of Alzheimer's disease risk. ***Nature***. 2022. DOI: 10.1038/s41586-021-04369-3. [†]Co-corresponding authors. [#]Contributed equally.
   a. News & Views: Transcriptomic mapping of the human cerebrovasculature. Ihara M, Yamamoto Y. https://doi.org/10.1038/s41582-022-00650-9.
   b. Research Briefing: Molecular map of the human blood–brain barrier reveals links to Alzheimer's disease. https://doi.org/10.1038/d41586-022-00350-w.
2. **Yang AC*,** Kern F*, Losada PM, Agam MR, Maat CA, Schmartz G, Fehlmann T, ... McNerney MW, Channappa D, Cobos I, Ludwig N, Schulz-Schaeffer WJ, Keller A[#], Wyss-Coray T[#].
   Dysregulation of brain and choroid plexus cell types with severe COVID-19. ***Nature***. 2021. https://doi.org/10.1038/s41586-021-03710-0. *Equal contribution, #Co-corresponding authors.
   a. Research Highlight: Single-cell transcriptomics reveals neuroinflammation in severe COVID-19. Lemprière S. https://doi.org/10.1038/s41582-021-00536-2
3. **Yang AC**, Stevens MY, Chen MB, Lee DP, Stähli D, Gate D, Contrepois K, Chen W, Iram T, Zhang L, ... Bertozzi CR, Luo J, Snyder MP, Elias JE, Quake SR, James ML, Wyss-Coray T.
   Physiological blood-brain transport is impaired with age by a shift in transcytosis. ***Nature***, 2020. https://doi.org/10.1038/s41586-020-2453-z.
   a. News & Views: Unexpected amount of blood-borne protein enters the young brain. Munji RN, Daneman R. https://doi.org/10.1038/d41586-020-01791-x
   b. Editor's Choice: Plasma proteins have a ticket to ride the blood-brain barrier. Davis AA. https://doi.org/10.1126/scitranslmed.abd3610
4. Chen MB*, **Yang AC***, Yousef H*, Lee D, Chen W, Schaum N, Lehallier B, Quakes SR[#], Wyss-Coray T[#].
   Brain Endothelial Cells Are Exquisite Sensors of Age-Related Circulatory Cues. ***Cell Reports***, 2020. https://doi.org/10.1016/j.celrep.2020.03.012. *Equal contribution, #Co-corresponding authors.
5. **Yang AC**, du Bois H, Olsson N, Gate D, Lehallier B, Berdnik D, Brewer KD, Bertozzi CR, Elias JE, Wyss-Coray, T.
   Multiple Click-Selective tRNA Synthetases Expand Mammalian Cell-Specific Proteomics. ***JACS***, 2018. https://doi.org/10.1021/jacs.8b03074.

Physicists meet Immunology – the 13th Physics of Cancer Symposium

The conference was held September 28th – 30th at the Center for Biotechnology and Biomedicine (BBZ), Leipzig

Revealing unifying principles and generating a mechanistic understanding of cancer constitutes a thrilling chance for a fundamental breakthrough in cancer treatment. To fight cancer, we must truly understand this incredibly diverse and complex disease. This comprises identifying general patterns of malignant tumor behavior that are dictated by the laws of physics and to combine it with our knowledge about their characteristic genetic modifications and molecular expression behavior. Identifying these physical patterns of tumor behavior, understanding them mechanistically, and ultimately translating them into novel approaches for cancer diagnosis and treatment is the goal of the research area "Physics of Cancer". In this regard, medical research profits from the development of this interdisciplinary research approach. The goal of this field is to identify general physical mechanisms behind the growth and disseminations of tumors in the human body, with a focus on the effect of passive and active forces acting on cell signaling, cell proliferation and cell migration.

The 13th International Symposium 'Physics of Cancer' has highlighted the latest developments in this field. Four focus sessions comprised traditional topics such as the biophysical properties of malignant cells and tissue while also highlighting novel bio- und nanotechnological approaches with direct clinical applications to cancer-immunology, cancer cell migration and the tumor microenvironment and nanoagents for targeted cancer cell manipulation. In addition to the founder of the annual meeting Josef Käs (Leipzig University), this year's conference was organized by Cornelia Monzel (HHU Düsseldorf), Ben Fabry (FAU Erlangen) Mareike Zink (Leipzig University) and Jörg Schnauß (Leipzig University).

To reach and attract a global audience, and to allow international contributions, the conference was held in a hybrid format. Most talks and posters were presented in person at the Leipzig University while some presenters had chosen a digital format to highlight their new research results. All talks were streamed to the online audience. The schedule was especially adjusted to meet the heterogeneous demands of the presenters. The continued international interest in our annual conference remained high with more than 100 participants from around the world.

There were 23 invited and 6 contributed talks selected from the submitted abstracts. Of all 29 speakers, 10 were female. 25 posters were submitted and displayed at this year's meeting (three online). The following sections highlight some particularly relevant talks and events from the different sessions and provide and excerpt-like summary of the conference.

First day:

The symposium started with a **session on cancer immunotherapy** and was opened by a contribution of the conference organizer **Cornelia Monzel** (HHU Düsseldorf) with the title "Physics of Cancer - From Fundamental Biophysics to Translational Research". She tied the fundamental (biophysics of cancer cells) and the applied (medical) side with a focus on cell signaling on the molecular scale by employing state of the art STED and FRET analyses as well as superparamagnetic nanoparticles.

Subsequently, **Helmut Hanenberg** (University Hospital Düsseldorf) spoke about "Genetic Engineering of Immune Effector Cells for Cancer Therapy" effectively opening the topic of cancer immunotherapy from a medical perspective to the other researchers. Helmut gave a nice overview of the development CAR-T cell therapy and a comprehensive introduction to the field from a clinical perspective. He further introduced the idea to develop similar strategies for solid tumors and discussed the several problems, which need to be overcome.

David Odde effectively bridged the gap between medical and physical research in his presentation "Differential Migration Mechanics and Immune Response of Glioblastoma Subtypes". David talked about the different

migration mechanisms and the immune response of glioblastoma subtypes. He presented his group's work on the progression of cancer using a state-of-the-art immunocompetent mouse model. He came to conclusion that mesenchymal glioma cells spread faster into the brain tissue compared to proneural cells. While the species containing mesenchymal tumors lived longer than the one with proneural tumors, which was supported by the immune response that includes the T cells- mediated autophagy, similar to human tumors.

The first day was concluded by a **conference buffet dinner** and an accompanying poster session. The highlight of the evening was the bestowal of the **young scientist award** (funded by the DGZ) for the best three posters, which were announced at the last day of the conference. The first price was awarded to **Jonas**

From right to left: B. Fabry, K. Hosseini, J. Naumann, J. Schnauß, C. Monzel

Naumann (Leipzig University, Germany) for his work and presentation on "Mechanical Properties of the Premature Lung". The second place was shared between **Daphne O. Asgeirsson** (ETH Zürich, Switzerland) and **Kamran Hosseini** (TU Dresden, Germany) for their poster presentations on "Magnetically Controlled Micro-Deformation of 3D Tumor Models for Mechanobiological in situ Studies" and "Skin Epithelial Cells Change their Mechanics and Proliferation upon Snail-Mediated EMT Signaling", respectively.

Second day:

The day was comprised of the focus session on **Cancer Cell Migration and the Tumor Microenvironment.**

Cynthia Reinhart-King (Vanderbilt University, USA) gave a much-applauded talk about the role of the cell metabolism in cancer development and progression. She reported about temporarily changing ATP levels for different situations of cell migration and invasion. Using different setups such as collagen matrices, channel setups and tumor spheroids, she showed that in collective migration leader cells are regularly replaced

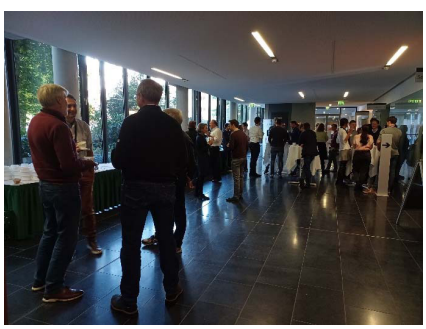
by follower cells, which could be related to cellular metabolic rates. Theoretical perspectives were presented by **Heiko Rieger** (Saarland University) in his talk about "Centrosome Positioning and Re-Positioning in Immune Cells" and **Ulrich Schwarz** (Heidelberg University) presenting "Control of Traction Forces, Force Propagation between Cells and Cell Migration by Optogenetics". Another impressive talk was given by **Adrian Shimpi** and **Garrett Beeghly** (Cornell University, USA) who talked about the biophysical contributions of adipose tissue to breast cancer invasion.

In the evening of the second conference day, all invited speakers were invited to the conference dinner at the "Café Cantona".

Third day:

The first session of this day was dedicated to **Nanoagents for Targeted Cancer Cell Manipulation**. **Simone Schürle-Finke** (ETH Zurich, Switzerland) talked about her aims to develop more targeted means for diagnostic and therapeutic interventions using engineered robots on a cellular level. She presented 3 distinct examples of magnetic micro- and nanorobots for biomedical applications. Especially the design of nanorobots controlled by magnetic fields was in the focus of her presentation. They can, for instance, trigger local enzyme release or can be used in the context of bacterial cancer therapy. **Young-wook Jun** (University of California San Franciso, USA) specifically focussed on spatio-temporal dynamics of Notch, a key cell communication receptor, and its signaling consequences in cells. By employing tools such as mechanogenetics, magnetically amplified protein-protein signals and single particle tracking they disentangled a long-standing mystery of how dynamic compartmentalization and colocalization of Notch creates enzymatically distinct environments.

After lunch, the day continued with the last **session on Cell Mechanics in Cancer**. The session was opened by **Denis Wirtz** (Johns Hopkins University, USA) describing his approach to probe large volumes (up to multi-centimeter cubes) of cancer tissue with high resolution based on the reconstruction of serially sectioned histological samples.



The algorithm labels distinct biological structures using an H&E stain and deep learning methods. The tissue visualization revealed cancer cells invading healthy tissue along the vascular and ductal system, which lead to the conclusion that cancer cells invade tissue along collagen alignment. **Peter Friedl** (The University of Texas, USA) presented experiments on invasion of the cancer cells under normoxia and hypoxia. Especially in hypoxia, cells undergo metabolic stress, which results in bioenergetic deprivation putting the tumor cells under adaptive responses in order to survive and migrate. He concluded that the mobility of the cells will be harder under hypoxia conditions because of the increase of the collagen fibres around the cells, while under normoxia, cells mobility is easier and their transition is ameboid. He emphasized that the migration and the nutrient deprivation of the cells are depending on the autophagy since it controls the unjamming of the cells.

The symposium included a mixture of talks from different scientific disciplines and provided new insights into cancer-related research that lead to lively discussions.

Author: Jörg Schnauß, Leipzig University

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UNIVERSITÄT LEIPZIG

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"Organoids" Meeting report of the 20th Workshop "Cell Biology of Viral Infections " of the German Society of Virology (GfV) in Schöntal, October 12th–14th, 2022

By Thomas Hoenen[1] and Eva Herker[2]

Group photo with 2022 workshop participants in front of the main building of the Cistercian Monastery Schöntal.

From October 12th to 14th 2022 the 20th Workshop "Cell Biology of Viral Infection" of the German Society of Virology (GfV) took place in the Schöntal Monastery near Würzburg. This year's special focus was "Organoids", represented by four keynote speakers from Germany, the U.K., Italy, and Spain, who highlighted different aspects of this topic, ranging from the history of organoid research and the development of various organoid systems, their use for studying diseases, and particularly also viral infections, all the way to cutting-edge biofabrication techniques.

In total the workshop had 39 on-site participants, among which were 30 students and postdocs. They actively participated in the workshop in 26 oral presentations in four sessions on Virus Entry, Replication and Morphogenesis, Virus Host Interactions, and Organoids. As in last years, there was ample discussion in the sessions, with a very active participation particularly of the younger attendees. Similarly, there was plenty of opportunity for networking at lunch and dinner gatherings, as well as at the well-attended social events.

The scientific program of the workshop started with a keynote lecture from Dr. Elena Martinez Fraiz from the Institute of Bioengineering of Catalonia in Spain. She spoke about the development and application of novel artificial matrices that mimic tissue micro and nanofeatures for use as biomimetics in *in vitro* assays. In particular, this encompassed biofabrication technologies such as bioprinting to develop complex in vitro models for small intestinal epithelium.

The second keynote lecture was given by Dr. Laura Pellegrini from the MRC Laboratory of Molecular Biology at the University of Cambridge, U.K. Dr. Pellegrini is a senior postdoctoral research fellow in the group of Madeline Lancaster, and recently was awarded a grant by the Wellcome Trust Foundation to start her own research group on organoids. As such she not only gave a fascinating talk on cerebral and choroid plexus organoids and their use to study neuroinvasion, but also served as an example for a young scientist at the stage of becoming an independent group leader, which of course was of great interest to the young virologists present at the workshop.

Dr. Veronica Krenn gave then a second lecture on cerebral organoids, complementing Dr. Pellegrini's talk. She recently transitioned from being a Marie Skłodowska-Curie postdoctoral fellow in the Knoblich laboratory at the Institute of Molecular Biotechnology in Vienna to being a principal investigator at the Department of Biotechnology and Biosciences of the University of Milan-Bicocca. The focus of this talk was on stem cell-derived 3D organoid cultures to develop human models for infectious diseases, and the study of the impact of viral infections and immunity on neural stem cell proliferation and brain development. Finally, while unable to attend in person, Dr Meritxell Huch from the Max Planck Institute of Molecular Cell Biology and Genetics in Dresden gave the last keynote lecture, highlighting the development of organoid research over time, and providing some background on her own work of developing liver and pancreas organoids. This represented an excellent example how organoids can serve as tools for studying complex cell biological processes. These keynote lectures were complemented by excellent presentations from all participants, covering a diverse range of topics and viruses. Like in the past years, at the end of the workshop all participants voted on the best oral presentation given by a young virologist, either student or junior postdoc. Silvia Albertini from the laboratory of Nicole Fischer at the University Medical Center Hamburg-Eppendorf, who had given a presentation on the development of skin organoids, was selected for the best oral presentation price.

This year also marked the end of the term of Dr. Eva Herker (Philipps-University Marburg) and Dr. Thomas Hoenen (Friedrich-Loeffler-Institut) as chairs of the working group "Cell Biology of Viral Infections" of the young GfV (jGfV), and thus also as organizers of this workshop. As successors, the participants voted for Dr. Gabrielle Vieyres from the Leibnitz Institute for Virology in Hamburg and Dr. Christian Sieben from the Helmholtz Centre for Infection Research in Braunschweig. They are already in the process of planning the 21st annual workshop, which will take place from October 18th to 20th at the Schöntal Monastery. More information and updates can be found on the workshop's website https://cellviro.g-f-v.org.

Finally, the organizers would like to thank the German Society for Virology (GfV), the German Society for Cell Biology (DGZ), and the companies ReBlikon and Singleron for their support. The workshop would not have been possible without their generous contributions!

Silvia Albertini, the winner of the prize for the best oral presentation of the 2022 workshop, together with the organizers Eva Herker and Thomas Hoenen.

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SAVE THE DATE

October 10–13, 2023

Cell Physics 2023

International DGZ Meeting

at **Saarland University**
Campus Saarbrücken

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German Society for Cell Biology (DGZ)
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