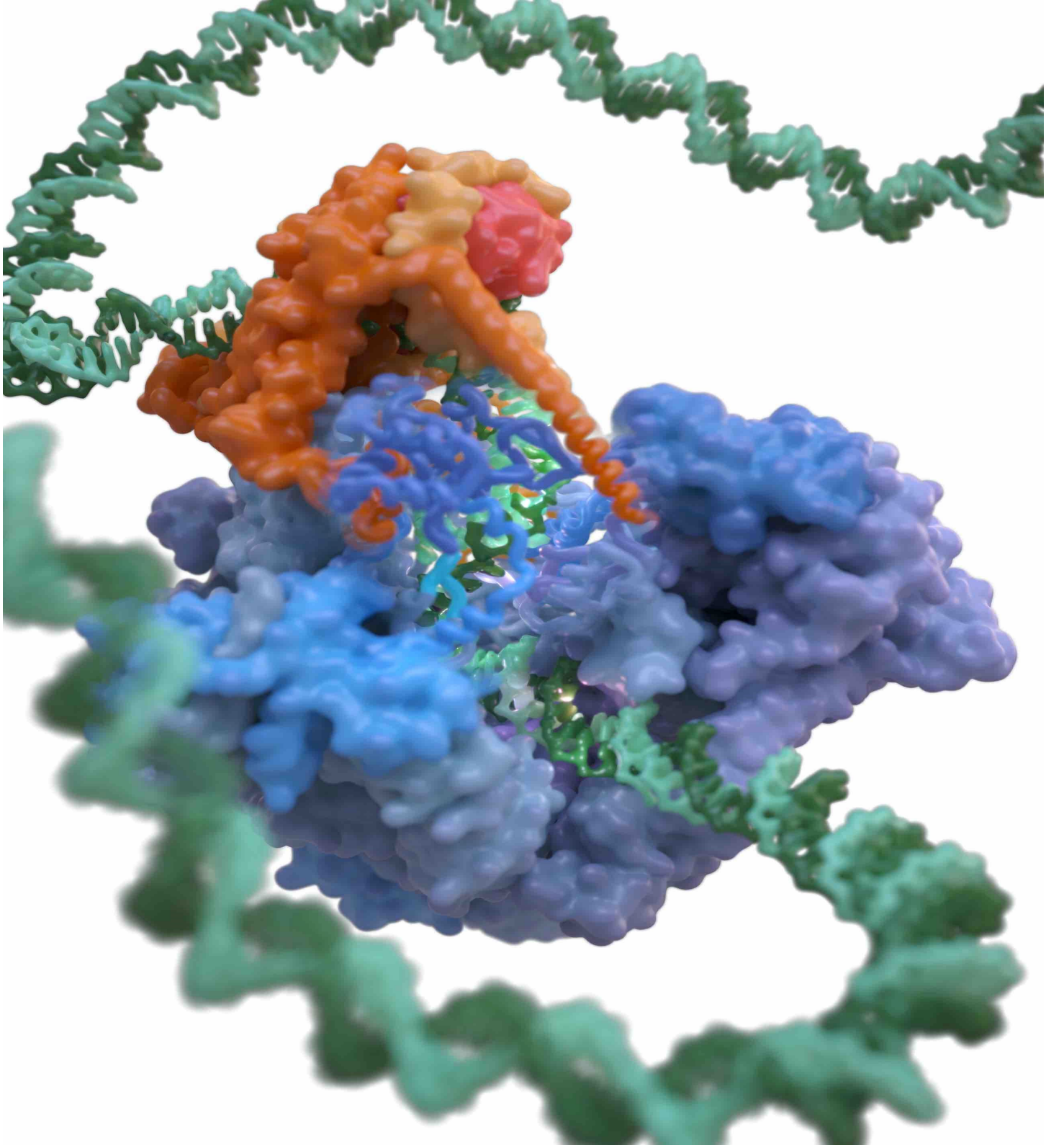

# *OddPols 2021*

*International Conference on*

*Transcription Mechanism and Regulation*

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## Guidance for Zoom Participation

Please see the guidance found on the conference web site (https://gs.ucdenver.edu/oddpols2021/) under “Information for Zoom Participation”

**COVER IMAGE:** “Structure of RNA Polymerase III pre-initiation complex”. Image is from Alessandro Vannini/Jeroen Claus (Phospho Biomedical Animation). The corresponding citation is:
Abascal-Palacios, G., Ramsay, E., Beuron, F. et al. Structural basis of RNA polymerase III transcription initiation. Nature 553, 301–306 (2018). https://doi.org/10.1038/nature25441


## Call for Summations and Papers

The OddPols 2021 meeting will be the basis for a Special Edition issue of the journal *GENE,* edited by Astrid Engel.

**1]** If you would like your presentation to be included (optional) and summarized accurately in the meeting report, please send the Title, Authors, and a short (100-- 200 word) summary to Astrid by July 15, 2021. We understand that the abstract in the book will not always accurately reflect the final detail in your actual presentation or that you may not wish to include some scientific details of unpublished work.

**2]** If you would like to publish an original research paper in this volume related to your presentation (though not necessarily identical), please submit your manuscript to *GENE* (http://www.journals.elsevier.com/gene/) by July 15, 2021, along with suggestions for appropriate reviewers. When submitting, select the option for the special issue (SI OddPols 2021: International Conference on Transcription Mechanism and Regulation) under "choose article type". Similarly, short communications, long or short review articles on the topic, and methodology reports are encouraged and desired. It would be best to check with Astrid on the topic beforehand to avoid substantial overlap.
For examples, see the 2012 and 2014 Special edition:
http://www.sciencedirect.com/science/journal/03781119/526/1
Details on format can be found here: https://www.elsevier.com/journals/gene/0378-1119/guide-for-authors

If you have any questions, feel free to contact Astrid (aengel@tulane.edu).

**Socialization Zoom Breakout Rooms** –

During the entirety of the conference (5 hours/day plus optional 6th hour) there will be 30 Zoom breakout rooms devoted to conversations. To go to one of these rooms, simply click on the "Breakout Rooms" icon at the bottom of the Zoom screen and choose the room you want to enter by clicking on the # to the right of it and entering the room. To go back to the main Zoom, just click the "leave room" button

These will be numbered as follows:
[1] Structure and Function of OddPols and Transcription machinery
[2] Regulation of transcription –activators and repressors
[3] Regulation of transcription –elongation and termination
[4] Chromatin and transcription, including gene silencing
[5] Transcription related to genome organization and nuclear structures
[6] OddPols in development, genetic disorders, cancer and disease
[7] Oddpols links to physiology, aging, and beyond

[8] Pol I
[9] Pol III
[10] Pol IV/V
[11] organelle Pols
[12] Archaeal Pols
[13] Other Pols
[14] New England Biolabs display and discussion during Breaks
[15] Meet the Editor -Publication and Reviewing Advice for Trainees, Sponsored by Developmental Dynamics (Wednesday Break, or by Appointment with Paul Trainor)

[16-30] fifteen numbered but unnamed Zoom rooms for discussion of whatever you like with whomever you like.

If you would like to meet another participant for a discussion in a Zoom room, the best tactic is likely to e-mail them which # room you would like to meet them and when. You could also put a private message to them in the Zoom Chat, but these are easy to miss if the recipient is not watching the chat. E-mails of conference participants are at the back of the Abstract book on the web site.

# OddPols 2021 Virtual Schedule

The times shown for the OddPols 2021 sessions reflect the Denver time zone (Mountain Daylight time). Each day the conference will begin promptly at 7:00 AM MDT.
Time Zone Conversions for Start Times:

| Denver | New York | London | Heidelberg | Shanghai | Canberra |
|---|---|---|---|---|---|
| 7:00 (AM) | 9:00 | 14:00 | 15:00 | 21:00 | 23:00 |

**Schedule of Sessions**
(12MT= 12 minute talks, 8MT= 8 minute talks, 3MT= special 3 minute talks)

## Day 1 (Monday, June 14)

**7:00: Introduction, Dave Engelke**

**7:07 Session 1 (12MT) Marv Paule, Chair**

| Abstract # | Speaker | Title |
|---|---|---|
| N/A | Marv Paule | History of OddPols |
| 15 | Kenneth A. Johnson | Kinetic and structural basis for inhibition of the SARS coronavirus RNA-dependent RNA polymerase by Remdesivir |
| 16 | Katsuhiko Murakami | Direct binding of TFEα opens DNA binding cleft of archaeal RNA polymerase |
| 17 | Simona Pilotto | The structural basis of RNA polymerase inhibition in archaea |

**8:00 Session 2 (3MT) Elaine Sanij, Chair**

| Abstract # | Speaker | Title |
|---|---|---|
| 19 | Natalia Zawrotna | Depletion of ribosome biogenesis proteins during yeast replicative aging |
| 20 | Christophe Dez | Investigating RNA Polymerase I regulation using a super-active mutant |
| 21 | Alana Belkevich | Exploring the interaction specificity between the eukaryotic RNA polymerase □-like subunits |
| 22 | Julia Daiß | Preparation of human RNA polymerase in close-to-native states |
| 23 | Florian B. Heiss | Conserved strategies of RNA polymerase I hibernation and activation |
| 24 | Nathan Munoff | Defining the critical DNA features targeted by RNA polymerase I Core Factor |
| 25 | Michael Pilsl | Structural basis of RNA polymerase I pre-initiation complex formation and promoter melting |
| 26 | Qianmin Wang | Structural insights into transcriptional regulation of human RNA polymerase III |
| 27 | Robyn Moir | Functional characterization of *Polr3a* hypomyelinating leukodystrophy mutations in the *S. cerevisiae* homolog, *RPC160* |

**9:00 Break - Zoom Breakout Rooms**

**10:00 Session 3 (8MT) Astrid Roy-Engel, Chair**

| Abstract # | Speaker | Title |
|---|---|---|
| 29 | Izabela Rudzińska | Defect in RNA polymerase III assembly in yeast *Saccharomyces cerevisiae* results in reprogramming of mRNA expression |
| 30 | Alexandria Cockrell | Transcription drives rDNA spatial organization and nucleolar morphology in fission yeast |
| 31 | Jorge Perez-Fernandez | Structure probing with MNase tethered to ribosome assembly factors provides insight into the structure of nascent pre-ribosomal RNA |
| 32 | Sui Huang | Nucleolar Homeostasis Connects with Nuclear Organization |
| 33 | Dalen Fultz | Assembly of Arabidopsis NOR sequences using ultra-long DNA sequencing and analysis of patterns of rRNA gene variation |
| 34 | Emiliana Weiss | Unveiling the variability and organization of the ribosomal RNA gene repeats with long sequencing technologies |

**11:00 Session 4 (12MT) Christoph Mueller, Chair**

| Abstract # | Speaker | Title |
|---|---|---|
| 36 | Magdalena Boguta | Biogenesis of yeast RNA polymerases III involves co-translational assembly mechanism |
| 37 | Carlos Fernández-Tornero | The role of RNA polymerase I in ribosomal DNA protection against UV light-induced DNA damage |
| 38 | Ruth Q. Jacobs | Defining the divergent enzymatic properties of the eukaryotic RNA polymerases |
| 39 | Akihito Fukudome | Structure of RNA-dependent RNA polymerase 2 and its implications for double-stranded RNA synthesis in RNA-directed DNA methylation |

**12:00 Social Hour in Zoom Breakout Rooms**

## Day 2 (Tuesday, June 15)

**7:00 Session 5 (12MT)** **Finn Werner, Chair**

| Abstract # | Speaker | Title |
|---|---|---|
| 41 | Rachel McNamar | Mammalian PAF49, the ortholog of the nonessential yeast RNA polymerase I subunit RPA34, is essential for rDNA transcription |
| 42 | Christoph Engel | Structural basis of RNA polymerase I transcription |
| 43 | Bruce A. Knutson | Molecular Topology of RNA Polymerase I Upstream Activation Factor |
| 44 | Tomasz W. Turowski | Functional analysis of RNA polymerase I reveals common features of transcription machinery |

**8:00 Session 6 (3MT)** **Bruce Knutson, Chair**

| Abstract # | Speaker | Title |
|---|---|---|
| 46 | Nisreen Chahid | Novel strategies for improving Pichia pastoris as an expression platform |
| 47 | Sebastian Kruse | Purification of native chromatin templates for investigation of RNA polymerase I promoter-specific transcription *in vitro* |
| 48 | Laura Martins | Transcriptional regulation of the four putative chromatin remodeling factors, CLSY1-4, in *Arabidopsis thaliana* |
| 49 | V. Miguel Palomar | The plastid chromatin organization is determined by protein binding |
| 50 | Katrin Schwank | Reconstitution of RNA polymerase I to investigate the function of the lobe binding subunits in *in vitro* assays |
| 51 | Rebecca Sizer | Using tRNA genes to improve the yield of therapeutic antibodies |
| 52 | Kanwal Tariq | Noncoding RNAs from human rDNA spacer regulate chromatin organization and accessibility |
| 53 | Thejaani Udumanne | Characterising changes in rDNA chromatin during malignant transformation |
| 54 | Guanghui Xu | Identifying novel factors that alter DNA methylation patterns in *Arabidopsis* |

**9:00 Break - Zoom Breakout Rooms**

**10:00 Session 7 (8MT)** **Ale Vannini, Chair**

| Abstract # | Speaker | Title |
|---|---|---|
| 56 | Michael Bartlett | Positioning of archaeal general transcription factors TFB and TFE during transcription initiation |
| 57 | Ewan Ramsay | Structural characterisation of human RNA polymerase III |
| 58 | Agata Misiaszek | Cryo-EM structures of human RNA polymerase I |
| 59 | Małgorzata Cieśla | The R3H domain-containing Rbs1 protein and the Upf1 helicase modulate the expression of Rpb10, a small subunit common to RNA polymerases |
| 60 | Gwenny Cackett | African Swine Fever Virus – from transcriptome to mechanism |

**11:00 Session 8 (12MT)  Rich Maraia, Chair**

| Abstract # | Speaker | Title |
|---|---|---|
| 62 | Dina Grohmann | DNA origami-based single-molecule force spectroscopy elucidates RNA Polymerase III pre-initiation complex stability |
| 63 | Francisco Gutiérrez-Santiago | Bud27 and its role in RNA pol III transcription |
| 64 | Nayef Jarrous | Coordination of transcription and processing of tRNA in human cells |
| 65 | Tom Moss | Mechanisms of rDNA promoter recognition and transcription initiation underly the UBTF neuroregression syndrome and the action of CX-5461 |

**12:00 Social Hour in Zoom Breakout Rooms**

## Day 3 (Wednesday, June 16)

**7:00 Session 9 (12MT)  Tom Santangelo, Chair**

| Abstract # | Speaker | Title |
|---|---|---|
| 67 | Mathias Girbig | Structural basis of RNA polymerase III transcription termination |
| 68 | Juanjuan Xie | Intrinsic and extrinsic mechanisms cooperate to ensure efficient termination of RNAPIII transcription |
| 69 | Saurabh Mishra | N terminal-and-Linker domain of the C11 subunit of RNA Polymerase III is necessary and sufficient for termination-associated reinitiation-recycling via interaction with C37/53 heterodimer |
| 70 | Tom Santangelo | FttA is a CPSF73 homologue that terminates transcription in Archaea |

**8:00 Session 10 (3MT)  Todd Blevins, Chair**

| Abstract # | Speaker | Title |
|---|---|---|
| 72 | Sara Javidnia | Population genetic analyses implicate biogenesis of translation machinery in human ageing |
| 73 | Cecelia Harold | High-content screen reveals lncRNAs as regulators of nucleolar form and function |
| 74 | Stephanie L. Cooper | *In vitro* characterization of RNA polymerase I inhibition by BMH-21 |
| 75 | Wenjun Fan | Widespread germline genetic heterogeneity of human ribosomal RNA genes |
| 76 | Stephanie Pitts | Identification of an E3 ligase regulating the catalytic subunit of RNA polymerase I |
| 77 | Neuton Gorjão | POLR1D, a common subunit of RNA polymerase I and III, influences its own expression |
| 78 | Kristin Watt | RNA Polymerase I and III function in neural crest cell and neuronal development |
| 79 | Jodie R. Malcolm | Widespread Association of ERα with tRNA genes in MCF-7 cells and primary breast tumors |
| 80 | Rita Ferreira | RNA polymerase I and II inhibitors act synergistically as cancer therapeutic |

**9:00 Break - Zoom Breakout Rooms**

**10:00 Session 11 (8MT)  Julie Law, Chair**

| Abstract # | Speaker | Title |
|---|---|---|
| 82 | Abu Musa Md Talimur Reza | Investigation of the effects of tRNA genes knock-out in human cells |
| 83 | Fabian Blombach | Cbp1 chromatinisation regulates transcription of CRISPR arrays |
| | | |

| | | |
|---|---|---|
| 84 | Breanna R. Wenck | Archaeal chromatin dynamics regulate the transcription apparatus |
| 85 | Andrew Loffer | Features of Pol IV-RDR2 dsRNAs dictate alternative DCL3-dicing patterns in the biogenesis of siRNAs guiding RNA-directed DNA methylation |
| 86 | Feng Wang | 23-nt siRNAs function as passenger strands for *Arabidopsis* AGO4-associated 24 nt siRNAs and are released by slicing |
| 87 | Dany Sibai | Transcription Termination Factor 1 (TTF1), a multifunctional regulator of ribosomal RNA gene activity and cell growth |

**11:00 Session 12 (12MT) Olivier Gadal, chair**

| **Abstract #** | **Speaker** | **Title** |
|---|---|---|
| 89 | Joachim Griesenbeck & Herbert Tschochner | The lobe binding subunits of RNA polymerase I cooperate to transcribe efficiently nucleosomal and non-nucleosomal templates |
| 90 | Ann-Kristin Östlund Farrants | Chromatin changes in the regulation of RNA pol I transcription |
| 91 | Guillermo Abascal-Palacios | Structural basis of Ty3 retrotransposon targeting of RNA polymerase III-transcribed genes |

**12:00 Social Hour in Zoom Breakout Rooms**

## Day 4 (Thursday, June 17)

**7:00 Session 13 (12MT)  Craig Pikaard, Chair**

| Abstract # | Speaker | Title |
|---|---|---|
| 93 | Todd Blevins | Evolution of Pol IV domains required for the functional docking of transposable element silencing factors |
| 94 | Andrzej Wierzbicki | Pervasive non-coding transcription as a genome surveillance mechanism |
| 95 | Julie Law | The CLASSY family controls tissue-specific DNA methylation patterns in Arabidopsis |
| 96 | Jeffrey Smith | The budding yeast rDNA locus and chromosome III share a common mechanism of condensin and Sir2 recruitment |

**8:00 Session 14 (3MT)  Christoph Engel, Chair**

| Abstract # | Speaker | Title |
|---|---|---|
| 98 | Alicja Armatowska | The connection between Maf1, a negative regulator of RNA polymerase III, and translation in yeast |
| 99 | Hitha Gopalan Nair | RNA polymerase III inhibition affects cytotoxic and tumour promoting effects of TNF |
| 100 | Aneta Jurkiewicz | MAF1 is involved in the regulation of RNA polymerase III activity in macrophages upon LPS treatment |
| 101 | Christopher Schächner | A system to study yeast RNA Polymerase I pre-initiation complex assembly *in vivo* – A “mini” story of cis-elements and transcription factors |
| 102 | Tamara Potapova | Anticancer compound library screen identifies Cdk inhibitors as novel inducers of nucleolar stress |
| 103 | Adriana Coke | Transcription by the Chloroplast-encoded RNA Polymerase May Determine Structural Organization of the Chloroplast Nucleoid |
| 104 | Hazel Mangan | NEAT; The combined power of NOR editing and chromosome transfer to study RNA polymerase I transcription, rRNA processing and human nucleoli |
| 105 | Anastasia McKinlay | Determination of ribosomal RNA gene repeat organization within Arabidopsis Nucleolus Organizer Regions by long-range sequencing and dotplot puzzle fitting |

**9:00 Break - Zoom Breakout Rooms**

**10:00 Session 15 (8MT)  Kate Hannan, Chair**

| Abstract # | Speaker | Title |
|---|---|---|
| 107 | Jessica Finlay-Schultz | A novel progesterone receptor-RNA polymerase III association represses estrogen-dependent growth in breast tumor patient-derived xenografts |

| | | |
|---|---|---|
| 108 | Ian Willis | System-wide changes in the metabolism of *Maf1* KO mice |
| 109 | Kevin Van Bortle | A cancer-associated RNA polymerase III identity drives expression of SNAR-A noncoding RNA |
| 110 | Trine Mogensen | Roles of POL III in antiviral defenses to varicella zoster virus and SARS-CoV2 |
| 111 | Alan C. Kessler | A dual function Pol III-dependent tRNA-gene and activation pathways of the cellular innate immune system |

**11:00 Session 16 (12MT) Craig Cameron, Chair**

| **Abstract #** | **Speaker** | **Title** |
|---|---|---|
| 113 | Shuping Zhong | The role and mechanism of pAMPKα-mediated dysregulation of Brf1 and RNA Pol III genes |
| 114 | Ashley Knox | RNA polymerase III transcribed gammaherpesvirus non-coding RNAs interact with host proteins and drive pathogenesis |
| 115 | Emilio Merheb | Defective myelination in an RNA polymerase III mutant leukodystrophic mouse |
| 116 | Stefanie Perrier | POLR3-related leukodystrophy: Defining novel phenotypes from very mild to extremely severe |

**12:00 Social Hour in Zoom Breakout Rooms**

## Day 5 (Friday, June 18)

**7:00 Session 17 (12MT)  David Schneider, Chair**

| Abstract # | Speaker | Title |
|---|---|---|
| 118 | Elaine Sanij | Inhibition of RNA Polymerase I Transcription Activates the DNA Damage Response and Demonstrates Therapeutic Efficacy in Ovarian Cancer |
| 119 | Paul Trainor | Ribosomopathies: congenital disorders of craniofacial and peripheral nervous system development and the potential for their prevention |
| 120 | Steven Zheng | SOD1 Regulates Ribosome Biogenesis in KRAS Mutant Non-Small Cell Lung Cancer |
| 121 | Katherine Hannan | Development of 2nd generation RNA Polymerase I inhibitors for cancer therapy |

**8:00 Session 18 (12MT)  Jennifer Gerton, Chair**

| Abstract # | Speaker | Title |
|---|---|---|
| 123 | Steve Bell | Chromosome Archae-tecture |
| 124 | Carson J. Bryant | High-throughput global analysis of miRNA drivers of ribosome biogenesis |
| 125 | Olivier Gadal | RNA polymerase I mutant affect ribosomal RNA processing and impact ribosomal DNA stability |
| 126 | Brian McStay | The formation and internal organisation of human nucleoli, revealed one NOR at a time |

**9:00 Break - Zoom Breakout Rooms**

**10:00 Session 19 (8MT)  Achim Griesenbeck, Chair**

| Abstract # | Speaker | Title |
|---|---|---|
| 128 | Abigail Huffines | Investigating the regulation of RNA polymerase I by the transcriptional activator Hmo1 |
| 129 | Soma Dash | Novel roles for RNA Polymerase I and associated factors in neural crest cell colonization of the gut and in the pathogenesis of Hirschsprung disease |
| 130 | Duy Khanh Phung | Structure, function and evolution of archaeal NusA paralogues |
| 131 | Kristin Scott | The Transcriptome-wide Distribution of Methyl-5 Cytosine in the Hyperthermophilic Archaeon, *Thermococcus kodakarensis* |
| 132 | Craig Marshall | Structural requirements for Eta-mediated archaeal transcription termination |

**11:00 Session 20 (12MT)  Linda Van Dyk, Chair**

| Abstract # | Speaker | Title |
|---|---|---|
| 134 | Nazif Alic | Odd Pols in fruit fly ageing |
| 135 | Ellen Busschers | A novel role for MAF1 and RNA pol III-dependent transcription in osteoblast differentiation and bone biology |
| 136 | Damian Graczyk | Manipulation of MAF1 levels affects pro-inflammatory functions of mouse macrophages |

**11:45 Closing Remarks  Ross Hannan**

**12:00 Social Hour in Zoom Breakout Rooms**

# ABSTRACTS

## Session 1, Chair: Marv Paule

**Kenneth A. Johnson** - "Kinetic and structural basis for inhibition of the SARS coronavirus RNA-dependent RNA polymerase by Remdesivir"

**Katsuhiko Murakami** - "Direct binding of TFEα opens DNA binding cleft of archaeal RNA polymerase"

**Simona Pilotto** - "The structural basis of RNA polymerase inhibition in archaea"

**Kinetic and structural basis for inhibition of the SARS coronavirus RNA-dependent RNA polymerase by Remdesivir**

**Tyler L. Dangerfield, Jack Bravo,**
**David Taylor and Kenneth A. Johnson***
Department of Molecular Biosciences
The University of Texas at Austin
2500 Speedway, Austin, TX 78712, USA

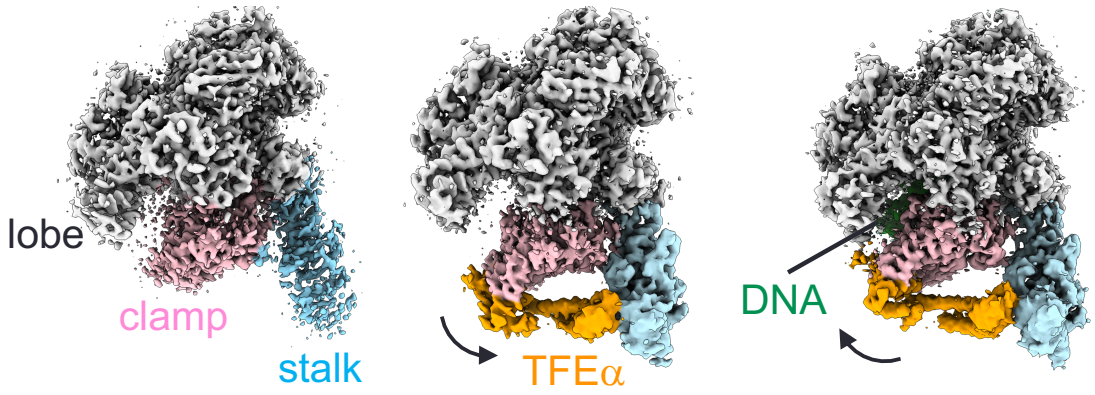
COVID-19 is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and is currently being treated using Remdesivir, a nucleoside analog that inhibits the RNA-dependent-RNA polymerase (RdRp). However, the enzymatic mechanism and efficiency of Remdesivir have not been determined and reliable screens for new inhibitors are urgently needed. Here we present our work to optimize expression of the polymerase in *E. coli*, followed by purification and kinetic analysis of an untagged NSP12/7/8 RdRp complex. Pre-steady-state kinetic analysis shows that our reconstituted RdRp catalyzes fast ($k_{cat}$ = 240—680 $s^{-1}$) and processive ($k_{off}$ = 0.013 $s^{-1}$) RNA polymerization. The specificity constant ($k_{cat}/K_m$) for Remdesivir triphosphate (RTP) incorporation (1.29 $\mu M^{-1}s^{-1}$) is higher than that for the competing ATP (0.74 $\mu M^{-1}$ $s^{-1}$). This work provides the first robust analysis of RNA polymerization and RTP incorporation by the SARS-CoV-2 RdRp and sets the standard for development of informative enzyme assays to screen for new inhibitors. We also solved a 3.9-Å-resolution cryoEM reconstruction of a remdesivir-stalled RNA-dependent RNA polymerase complex, revealing full incorporation of three copies of remdesivir monophosphate (RMP) and a partially incorporated fourth RMP in the active site. The structure reveals that RMP blocks RNA translocation after incorporation of three bases following RMP, resulting in delayed chain termination, which can guide the rational design of improved antiviral drugs. The SARS coronavirus is unusual for RNA viruses in the size (30 kb) and complexity of its genome and the RdRp responsible for its replication. Unlike RdRps from Hepatitis C virus or Polio Virus, the SARS-CoV-2 RdRp includes a subunit with 3'-5' proofreading activity. In future studies we will work to reconstitute the RdRp with the proofreading subunit, establish the overall fidelity of the polymerase and quantify the role of the proofreading activity in inhibition by Remdesivir.

**Direct binding of TFEα opens DNA binding cleft of archaeal RNA polymerase**

Sung-Hoon Jun[1,2,3], Jaekyung Hyun[1,4], Jeong Seok Cha[2], Hoyoung Kim[2], Michael S. Bartlett[5], Hyun-Soo Cho[2], Katsuhiko S. Murakami[3*]
[1]Electron Microscopy Research Center, Korea Basic Science Institute, Chungcheongbukdo 28119, Republic of Korea; [2]Department of Systems Biology, College of Life Science and Biotechnology, Yonsei University, Seoul 03722, Republic of Korea; [3]Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA 16802, USA (http://rnaplab.org); [4]Molecular Cryo-electron Microscopy Unit, Okinawa Institute of Science and Technology Graduate University, Okinawa 904-0495, Japan; [5]Department of Biology, the Portland State University, Portland, OR 97207, USA.

Opening of the DNA binding cleft of multi-subunit form of cellular RNA polymerase (RNAP) is necessary for promoter DNA binding, loading and opening DNA around the transcription start site and transcription initiation, but the underlying molecular mechanism of the cleft opening and closing of the stalk-containing archaeal and eukaryotic RNAPs is not structurally characterized. Here, we report on the cryo-electron microscopy structures of the RNAP, RNAP-TFEα binary, and RNAPTFEα-promoter DNA ternary complexes from archaea, *Thermococcus kodakarensis* (Tko). The structures reveal that TFEα bridges the RNAP clamp and stalk domains to open the DNA binding cleft. Positioning of promoter DNA into the cleft closes it while maintaining the TFEα interactions with the RNAP mobile modules. The structures and photo-crosslinking results also suggest that the conserved aromatic residue in the extended winged-helix domain of TFEα interacts with promoter DNA to stabilize the transcription bubble. This study provides a structural basis for the functions of TFEα and elucidates the mechanism by which the DNA binding cleft is opened during transcription initiation in the stalk-containing RNAPs.

Jun, S.-H. et al. The X-ray crystal structure of the euryarchaeal RNA polymerase in an open-clamp configuration. *Nat Commun* **5**, 5132–11 (2014).
Jun, S.-H. *et al*. Direct binding of TFEα opens DNA binding cleft of RNA polymerase. *Nat Commun* **11**, 6123 (2020). (https://www.youtube.com/watch?v=g_MCXnnq6gk)



**The structural basis of RNA polymerase inhibition in archaea**

Simona Pilotto[1,*], Thomas Fouqueau[1], Natalya Lukoyanova[2], Carol Sheppard[3], Alan C. M. Cheung[4], and Finn Werner[1]

[1]RNAP laboratory, Institute for Structural and Molecular Biology, University College London, Gower Street, WC1E 6BT London, United Kingdom; [2]Institute for Structural and Molecular Biology, Birkbeck College, Malet Street WC1E 7HX London, United Kingdom; [3]Section of Virology, Department of Infectious disease, Imperial College London, W2 1PG, London, United Kingdom; [4]School of Biochemistry, University of Bristol, Bristol, BS8 1TD, United Kingdom

The inhibition of RNA polymerases activity plays an important role in the regulation of transcription in response to environmental changes and in the virus-host relationship. Here, we present the cryo-EM structures of two such RNAP-inhibitor complexes that provide the structural basis underlying RNAP inhibition in archaea. The Acidianus Two-tailed Virus (ATV) encodes the RIP factor, a 15 kDa helical bundle that binds tightly to the clamp and rudder elements in the DNA-binding channel [1]. RIP inhibits RNAP via occlusion of binding sites for transcription initiation (TFB) and elongation factors (Spt4/5) and by preventing the loading of the promoter template DNA strand into the active site of RNAP. Infection with the Sulfolobus Turreted Icosahedral Virus (STIV) induces the expression of the host factor TFS4, a paralog of the transcript cleavage factor TFS1 which has been shown to stop cell growth [2]. In contrast to RIP, TFS4 is a potent allosteric effector, its binding inside the secondary channel displaces the trigger loop and induces a widening of the DNA-binding channel of RNAP which leads to the unwinding of the bridge helix. Importantly, the conformational changes induced by TFS4 are closely related to catalytically inactivated states of RNAP found in different domains of life such as dimeric Pol I in eukaryotes and the RNAP-Gfh1 complex in bacteria.

[1] Sheppard, C. et al. Repression of RNA polymerase by the archaeo-viral regulator ORF145/RIP. Nat Commun (2016).

[2] Fouqueau, T. et al. The transcript cleavage factor paralogue TFS4 is a potent RNA polymerase inhibitor. Nat commun (2017).

# Session 2, Chair: Elaine Sanij

**Natalia Zawrotna** - "Depletion of ribosome biogenesis proteins during yeast replicative aging"

**Christophe Dez** - "Investigating RNA Polymerase I regulation using a super-active mutant"

**Alana Belkevich** - "Exploring the interaction specificity between the eukaryotic RNA polymerase alpha-like subunits"

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**Michael Pilsl** - "Structural basis of RNA polymerase I pre-initiation complex formation and promoter melting"

**Qianmin Wang** - "Structural insights into transcriptional regulation of human RNA polymerase III"

**Robyn Moir** - "Functional characterization of Polr3a hypomyelinating leukodystrophy mutations in the S. cerevisiae homolog, *RPC160*"

**Depletion of ribosome biogenesis proteins during yeast replicative aging**

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*Saccharomyces cerevisiae* replicative lifespan (RLS) is defined as the number of divisions that individual mother cells undergo before losing viability. Aging mother cells accumulate age-related factors that are not inherited by daughter cells, such as extrachromosomal rDNA circles (ERCs) that are produced through mitotic rDNA recombination. Overall instability of the rDNA and the accumulation of ERCs in mother cells have both been implicated in the replicative aging process. We are interested in determining the mechanisms by which the rDNA initially becomes unstable during the early stages of aging, prior to the accumulation of ERCs. The Smith lab recently identified several nuclear factors required for maintaining rDNA stability that are strongly depleted from mother cells during the aging process, as was previously described for Sir2. These factors include Sir2 interacting partners such as Sir4 and Net1, the cohibin complex (Lrs4 and Csm1), and several subunits of the cohesin complex. Such proteins are potential dosage-dependent longevity factors, which we have already shown for the Mcd1 subunit of cohesin. We hypothesized that identification of additional age-depleted nuclear proteins, especially those associated with the nucleolus, would facilitate mechanistic analysis of genomic instability associated with aging. To quantitatively analyze nuclear protein abundance, we isolated nuclei from young (~1 bud scar) and replicatively aged (and biotinylated) mother cells (~8 bud scars) that were separated after 24 hrs of growth in mini-chemostats surrounded by ring magnets. We then performed quantitative mass spectrometry using tandem mass tag (TMT)-labeled nuclear extracts from the young and old cell populations. Here, we present the analysis of results from our screen using tools such as STRING, SGD, and Gene Ontology (GO) term analysis. Interestingly, the top Gene Ontology (GO) terms for age-depleted proteins were related to ribosome biogenesis, including RNA Pol I and Pol III transcription, rRNA processing, and ribosome assembly, all of which occur in the nucleolus. Future investigation will determine whether such nucleolar changes are a consequence or causative of aging.

## Investigating RNA Polymerase I regulation using a super-active mutant

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RNA Polymerase I (Pol I) is the enzyme responsible of pre-ribosomal RNA synthesis. Despite producing a single transcript, Pol I is by far the most active RNA polymerase, responsible for 60% of total transcriptional activity in exponential growing cells. Nevertheless and despite its central importance for cell metabolism, mechanistic understanding *in vivo* rRNA production regulation by Pol I remains sparse. However, it is known that, in order to adapt its ribosome production in function of metabolism requirement, cells display a large panel of regulation elements aiming at producing more or less ribosomes. In this study, we take advantage of a super-active Pol I mutant we previously identified in *S. cerevisiae* (Darrière et al. 2019), hereafter named SuperPol I, to investigate Pol I transcription regulation. Indeed, we show this mutant displays the ability to produce *in vivo* two-fold more rRNA than WT Pol I. Moreover, we show that SuperPol I is resistant to BMH-21 treatment. BMH-21 is a small molecule that inhibits Pol I transcription and induces degradation of the two largest subunits of the WT enzyme: Rpa190 and Rpa135 (Wei et al. 2018). In contrast, SuperPol I is stabilized in cells treated with BMH21 and Pol I transcription is maintained. All together, these results allowed us to hypothesize that WT Pol I bears intrinsic inhibitory elements of the transcription. Preliminary results tends to show that WT Pol I is subjected to premature termination at 5' end of the ribosomal DNA gene, thus reducing its processivity compared to SuperPol I.

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**Exploring the interaction specificity between the eukaryotic RNA polymerase α-like subunits**

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DNA-dependent RNA Polymerases (Pols) are present in every living cell and even encoded by some viruses. Pols are responsible for the process of transcription, and Pols from the three domains of life are constructed of a conserved core complex that includes a dimer of α or α-like subunits that serve as a scaffold for Pol assembly. Bacteria and archaea each encode a single Pol that transcribes all forms of RNA, while eukaryotes encode three specialized Pols (I-III) containing one of two distinct α-like heterodimers. One α-like heterodimer is shared between Pols I and III, while there is a paralogous Pol II heterodimer. The α-like subunits are clinically relevant as mutations in the Pol I/III heterodimer are associated with Treacher Collins Syndrome and 4H Leukodystrophy, while mutations in the Pol II heterodimer are associated with Primary Ovarian Insufficiency. These mutations often result in defects in Pol assembly and/or activity. It is currently unclear if heterodimer formation is functionally similar in α-like subunit orthologs and/or paralogs. To test this, we mutated several regions of the yeast and human α-like subunits to test their contribution to heterodimer interaction. Here we show that different regions serve differential roles in heterodimerization, in a polymerase- and species-specific manner. This suggests that although the subunits are evolutionary conserved, they interact differently. More broadly, these findings help explain why some disease mutations have little to no effect in yeast and possibly other model systems, and may inform us how to make better disease models.

## Preparation of human RNA polymerase in close-to-native states

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In eukaryotes, RNA Polymerase (Pol) III is specialized for the transcription of tRNAs and other short, untranslated RNAs. Pol III is a determinant of cellular growth and lifespan across eukaryotes. Upregulation of Pol III transcription is observed in cancer and causative Pol III mutations have been described in neurodevelopmental disorders and hypersensitivity to viral infection. However, structure-function analysis was limited to yeast Pol III in which many features are not identical. To obtain a highly purified, yet functionally active human Pol III for *in vitro* analysis, we employed CRISPR/Cas9 genome editing to create a homozygous knock-in of a cleavable green fluorescent protein (GFP)-tag at the C terminus of subunit RPAC1 (shared between Pol I and Pol III). Cellular fractionation experiments followed by immuno-purification using an anti-GFP nanobody revealed that Pol III is present in both nuclear and cytoplasmic fractions. Large-scale purification from suspension cultures enabled the isolation of human Pol III from total cell extracts with yields and quality suitable for functional and structural studies. Our approach enables structural analysis by single-particle cryo-Electron Microscopy to compare Pol III enzymes from yeast to human and allowing the mapping of most reported genetic mutations.

Ramsay EP*, Abascal-Palacios G*, Daiß JL*, King H, Gouge J, Pilsl M, Beuron F, Morris E, Gunkel P, Engel C[$], Vannini A[$]. Structure of human RNA polymerase III. *Nat Commun.* 2020 Dec 17;11(1):6409. doi: 10.1038/s41467-020-20262-5
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**Conserved strategies of RNA polymerase I hibernation and activation**

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RNA polymerase (Pol) I transcribes the ribosomal RNA precursor in all eukaryotes. Structures of monomeric states and of actively elongating Pol I from *Saccharomyces cerevisiae* (*Sc*) were solved by single particle Electron Cryo Microscopy (cryo-EM), revealing that the cleft of Pol I contracts upon activation. Inactive Sc Pol I dimers form dependent on the organism-specific 'connector' domain and feature a widely expanded active center cleft.
To understand whether the mechanisms 'inactivation by dimerization' and 'activation by contraction' are shared among organisms, we use single particle cryo-EM to determine the structure of Pol I from *Schizosaccharomyces pombe* (*Sp*). High-resolution 3D reconstructions of the monomeric and an actively elongating form show that Pol I cleft contraction upon transcription activation is common to both organisms. Although preparations contain all 14 subunits of *Sp* Pol I, cryo-EM density is lacking for the heterodimeric sub-complex A49/A34.5 in both reconstructions. Furthermore, we show that *Sp* Pol I can dimerize *in vitro* and solve the cryo-EM reconstruction of a Pol I dimer. Comparative modelling shows that *Sp* Pol I dimer architecture diverges from the *Sc* counterpart and that dimer formation is independent of the A43 connector domain. Our results indicate that the regulatory principles 'activation by contraction' and 'hibernation by dimerization' may be shared among organisms despite structural divergence of involved elements.

Heiss, F. B., Daiß, J. L., Becker, P., & Engel, C. Conserved strategies of RNA polymerase I hibernation and activation. *Nature Communications*, 12(758), 1-9 (2021).

**Defining the critical DNA features targeted by RNA polymerase I Core Factor**

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A critical and essential Pol I transcription factor in yeast is Core Factor (CF) which binds to a ~24 bp region in the rDNA promoter called the Core Element (CE). CF plays fundamental roles in the Pol I transcription process helping to recruit Pol I and open Pol I promoter DNA before initiation. However, it is not yet known how CF is precisely recognizes the CE. When interacting with DNA, proteins us two main mechanisms: i) base-pair readout, and ii) shape/structural readout. Base-readout is the most common and is dominated by hydrogen bonding between the amino acid residues and base-pair hydrogen bond donors and acceptors. The second mechanism centers around DNA shape readout where proteins target specific DNA features such as curvature, bendability, and groove width. Previous studies from our lab have shown that CF and its human orthologue, Selectivity Factor 1 (SL1), use an evolutionarily conserved mechanism to target DNA which is governed by interactions with the GC minor groove, a unique surface rarely targeted by DNA-binding proteins. To better understand the extent of structural recognition as well as what specific DNA features CF may be using to interact with CE, we have employed a variety of selection-based methods to resolve the rules governing CF's interaction with DNA. Our findings are consistent with a model that CF-CE interaction is governed primarily by DNA shape based structural features rather than sequence.

**Structural basis of RNA polymerase I pre-initiation complex formation and promoter melting**

Michael Pilsl* & Christoph Engel

In fast growing cells, up to 60% of total transcription is devoted to ribosomal RNA (rRNA) synthesis. In eukaryotes a specialized enzyme, RNA polymerase I (Pol I), synthesizes a polycistronic precursor rRNA which is the 35S rRNA in the yeast *S. cerevisiae*. Whereas Pol II and Pol III use similar mechanisms to initiate transcription, the processes underlying Pol I promoter recognition, initiation complex formation and DNA melting substantially diverge. Transcription initiation requires CF (core factor), Pol I and the initiation factor Rrn3. We obtained a high-resolution cryo-EM reconstruction of a Pol I early initiation intermediate assembled on a double-stranded promoter scaffold that prevents the establishment of downstream DNA contacts. Our analyses demonstrate how efficient promoter-backbone interaction is achieved by combined re-arrangements of flexible regions in the CF subunits Rrn7 and Rrn11. Destabilization of the melted DNA region correlates with a contraction of the polymerase cleft upon transcription activation, thereby combining promoter recruitment with DNA melting. The results outline the divergence of steps in Pol I initiation from other transcription systems.

Pilsl, Michael; Engel, Christoph (2020): Structural basis of RNA polymerase I pre-initiation complex formation and promoter melting. In: *Nat Comms* 11 (1), S. 1206. DOI: 10.1038/s41467-020-15052-y.

**Structural insights into transcriptional regulation of human RNA polymerase III**

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RNA polymerase III (Pol III) synthesizes structured, essential small RNAs, such as transfer RNAs (tRNAs), the 5S ribosomal RNA (rRNA) and the U6 small nuclear RNA (snRNA). Deregulation of Pol III has been directly connected to cancer development . Pol III is the largest RNA polymerase with a conserved core region and eight constitutive regulatory subunits. However, how these factors regulate Pol III transcription remains unclear. Here, we determined cryo-electron microscopy structures of human Pol III in both apo and elongating states. The structures unveil an unexpected apo state in which the RPC7 tail occupies the DNA-RNA-binding cleft of Pol III, suggesting that RPC7 plays important roles in both autoinhibition and transcription initiation. Pol III possesses an orchestrated movement during the apo-to-elongating transition, in which the RPC7 couples the majority of the conformational changes together upon DNA-RNA hybrid association. The structures also reveal a constant proofreading mechanism by TFIIS-like subunit RPC10 that stably stays in its catalytic position in the secondary channel, explaining the high fidelity of Pol III transcription. Our work provides an integrated picture of the mechanistic understanding of Pol III transcription regulation.

**Functional characterization of *Polr3a* hypomyelinating leukodystrophy mutations in the *S. cerevisiae* homolog, *RPC160***

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Mutations in RNA polymerase III (Pol III) cause hypomeylinating leukodystrophy (HLD) and neurodegeneration in humans. POLR3A and POLR3B, the two largest Pol III subunits, together form the catalytic center and carry the majority of disease alleles. Disease-causing mutations include invariant and highly conserved residues that are predicted to negatively affect Pol III activity and decrease transcriptional output. A subset of HLD missense mutations in POLR3A cluster in the pore region that provides nucleotide access to the Pol III active site. These mutations were engineered at the corresponding positions in the *Saccharomyces cerevisiae* homolog, Rpc160, to evaluate their functional deficits. None of the mutations caused a growth or transcription phenotype in yeast. Each mutation was combined with a frequently occurring pore mutation, POLR3A G672E, which was also wild-type for growth and transcription. The double mutants showed a spectrum of growth phenotypes from wild-type to lethal, with only the least fit combinations showing an effect on Pol III transcription. The growth phenotype was sensitive to a modifier of translation in the genetic background. Analysis of Pol III transcription in vivo in one double-mutant strain (KE) showed a global defect in tRNA synthesis that did not affect the steady-state level of mature tRNAs. Synthesis of only a subset of other Pol III-transcribed genes was defective, specifically RPR1 RNA (the RNA component of nuclear RNase P) and SNR52 snoRNA (a rRNA methylation guide). Affinity-purified KE Pol III was broadly defective in both factor-independent and factor-dependent transcription in vitro across genes that represent the yeast Pol III transcriptome. The biochemical properties of KE Pol III suggest that it partitions into two forms: one as competent for transcription as wild-type Pol III and the other form, functionally inactive. Together, these analyses suggest that the effect of background modifiers of Pol III transcription and gene-specific sensitivity to alterations in Pol III activity likely contribute to transcription defects caused by HLD mutations in patients.

Robyn D Moir, Christian Lavados, JaeHoon Lee and Ian M Willis. Functional characterization of *Polr3a* hypomyelinating leukodystrophy mutations in the *S. cerevisiae* homolog, *RPC160*. *Gene.* 2021 Feb 5;768:145259. Epub 2020 Oct 22.

# Session 3, Chair: Astrid Roy-Engel

**Izabela Rudzińska** - "Defect in RNA polymerase III assembly in yeast Saccharomyces cerevisiae results in reprogramming of mRNA expression"

**Alexandria Cockrell** - "Transcription drives rDNA spatial organization and nucleolar morphology in fission yeast"

**Jorge Perez-Fernandez** - "Structure probing with MNase tethered to ribosome assembly factors provides insight into the structure of nascent pre-ribosomal RNA"

**Sui Huang** - "Nucleolar Homeostasis Connects with Nuclear Organization"

**Dalen Fultz** - "Assembly of Arabidopsis NOR sequences using ultra-long DNA sequencing and analysis of patterns of rRNA gene variation"

**Emiliana Weiss** - "Unveiling the variability and organization of the ribosomal RNA gene repeats with long sequencing technologies"

**Defect in RNA polymerase III assembly in yeast *Saccharomyces cerevisiae* results in reprogramming of mRNA expression**

Izabela Rudzińska[1*], Małgorzata Cieśla[1], Tomasz W Turowski[2], Alicja Armatowska[1], Ewa Leśniewska[1] and Magdalena Boguta[1]

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Interplay between nuclear RNA polymerases is key to growth control. By using RNAseq we explored the ways in which mRNA transcription is influenced by a defect in the biogenesis of Pol III in yeast *Saccharomyces cerevisiae*. We used the cold-sensitive mutant *rpc128-1007*, which prevents assembly of the Pol III complex and consequently leads to low tRNA levels. mRNA upregulation in *rpc128-1007* cells was generally stronger and involved more genes than downregulation. The observed induction of mRNA expression was mostly indirect and resulted from the de-repression of general control transcription factor Gcn4. mRNA genes that were downregulated by the reduction of Pol III assembly comprise the proteasome complex. We also investigated the ways in which the reprogramming of Pol II genes is influenced by the *rpc128-1007* suppressors *RBS1* and *PRT1*, which encode the Pol III assembly factor and the subunit of translation initiation factor eIF3, respectively. Both of the suppressor genes countered the effects of *rpc128-1007* on the expression of Gcn4-dependent genes and the effects of *PRT1* were stronger than the effects of *RBS1*. Additionally, Rbs1 modulates Gcn4 activity in a manner that depends on of the Pho85 cyclin Pcl5. We have shown that the downregulation of Pcl5 protein levels by Rbs1 overproduction leads to a Gcn4 response that is likely related to the stabilization of Gcn4 protein. Altogether, our data contribute to the regulatory network which links transcription of different RNA classes. This work was supported by the National Science Centre [UMO-2017/25/B/NZ1/01889].

**Transcription drives rDNA spatial organization and nucleolar morphology in fission yeast**

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To keep up with the demands of ribosome biogenesis, ribosomal DNA (rDNA) loci are the most highly transcribed regions of the genome. rDNA transcription is required for the formation of a nuclear compartment called the nucleolus, where several steps of ribosome biogenesis occur. Nucleolar structure is altered in many human diseases including more than 20 types of cancer. The prevalence of altered nucleolar morphology in human disease demands an understanding of how aberrant nucleolar structure arises and how it relates to disease pathogenesis. Our hypothesis is that nucleolar morphology is driven by the activity and organization of the rDNA loci. To test this hypothesis, we have developed a new approach to visualize and quantify rDNA spatial organization in fission yeast. We have characterized a GFP-tagged bacterial DNA-binding protein called GapR as a new marker for rDNA analysis in live cells. We can quantify rDNA spatial organization and nucleolar morphology across the cell cycle by combining GapR-GFP expression with an mCherry-tagged nucleolar marker. Using yeast genetics and pharmacological treatments to target the ribosome biogenesis pathway, we have discovered how rDNA transcriptional activity impacts its spatial organization. We find that transcriptional inactivity of rDNA loci results in rDNA condensation and nucleolar compaction, while increased transcription results in increased rDNA volume and nucleolar expansion. Our studies provide the first in-depth analysis of how rDNA activity regulates rDNA spatial organization and nucleolar morphology, enabled by our unique rDNA imaging marker. Understanding how the organization of these fundamental nuclear structures is regulated in fission yeast brings us a step closer to interpreting aberrant nucleolar morphologies in human disease.

## Structure probing with MNase tethered to ribosome assembly factors provides insight into the structure of nascent pre-ribosomal RNA

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The synthesis of ribosomes involves the correct folding of the pre-ribosomal (r)RNA within pre-ribosomal particles. The early steps of ribosome synthesis take place on the nascent transcript to produce the first ribosomal precursor, known as the small subunit (SSU) processome. At these early stages, the pre-ribosomal particles undergo structural and compositional changes resulting in heterogeneous populations of particles with highly flexible regions. Structure probing methods are suitable to resolve these structures and provide evidence about the architecture of ribonucleoprotein complexes. In addition, MNase tethered to RNA-associated proteins provides unique clues about the RNA's flexibility independently of the secondary structure. In our study, we found a large population of nascent pre-ribosomal RNAs, which have not been identified previously. Our structure probing analyses elucidate the relative organization of different regions from both the nascent pre-rRNA population and the U3 snoRNA within the SSU processome. Finally, our structural approach provides formal evidence for the physical proximity between the small and large subunit precursors, which supports our published model for the balanced control of both ribosomal subunits[*].

* Braun CM, Hackert P, Schmid CE, Bohnsack MT, Bohnsack KE, Perez-Fernandez J. Pol5 is required for recycling of small subunit biogenesis factors and for formation of the peptide exit tunnel of the large ribosomal subunit. Nucleic Acids Res. 2019;48: 405–420. doi:10.1093/nar/gkz1079

**Nucleolar Homeostasis Connects with Nuclear Organization**

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Beyond its classically established role in ribosome synthesis, the nucleolus is now known to be a multi-functional nuclear organelle. Although certain stressors and genotoxic encounters disrupt nucleolar integrity and ribosome synthesis, they can influence much more than just the nucleolus, making it difficult to selectively address the nucleolar function. For example, classical studies use low concentrations of acintomycin D to inhibit rDNA transcription and perturb the nucleolus. But the drug also inhibits topoisomerases I and II and intercalates DNA with selective affinity for G+C-rich sequences that abound throughout the genome. To more selectively tease out the potential unrecognized roles of the nucleolus, we disrupted rDNA transcription by siRNA knockdown of the RNA polymerase I largest subunit, RPA194, in HeLa cells, which reduced pre-rRNA synthesis and induced nucleolar segregation, similar to that observed in actinomycin D-treated cells. Nucleolar segregation induced by RP194 knockdown led to a repositioning of the centromeric regions of chromosomes normally situated in the nucleolar periphery. In addition, spatially distal Cajal bodies underwent morphological alterations and loss of certain components. Furthermore, certain genomic loci situated far from nucleoli displayed extensive repositioning. These widespread effects throughout the 3-D nucleome were not observed when the pre-ribosomal RNA processing factor UTP4 was knocked down, which also reduced ribosome synthesis, but does not induce nucleolar segregation, establishing that the RPA194 effects throughout the nucleus are not due to an inhibition of ribosome synthesis but rather a nucleolar reorganization. These findings point to an intranuclear commutative system that links the homeostasis of the nucleolus to the maintenance and localization of certain proximal and distal nuclear bodies and gene loci.

**Assembly of Arabidopsis NOR sequences using ultra-long DNA sequencing and analysis of patterns of rRNA gene variation**

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Nucleolus Organizer Regions (NORs) are composed of long tandem arrays of 45S rRNA genes and are the sites of RNA polymerase I transcription in the nucleus. The minimal sequence diversity among individual rRNA genes limits the usefulness of short-read sequencing technologies for determining gene order within these tandem arrays. Thus, reconstruction of the sequence of entire NORs is currently limited, leaving multi-million basepair gaps in eukaryotic genome assemblies.

Long-read sequencing technologies, such as Oxford Nanopore Technology (ONT) sequencing and Pacific Bioscience's SMRT sequencing, have vastly improved the ability to assemble chromosomal regions consisting of repetitive DNA. However, even the longest reported sequencing reads, derived from ONT sequencing, are insufficient to span entire NORs. Nonetheless, subtle sequence variation among individual rRNA gene copies suggests that it may be possible to build contigs from long reads that span several rRNA genes. We are attempting to do so in *Arabidopsis thaliana*, which has two NORs each composed of 400 or more rRNA genes that are each ~10 kb in size. In doing so, two challenges quickly emerged: 1) the sequence variation between 45S copies is far smaller than the error rate of ONT sequencing and 2) distinct rRNA gene variants are present in multiple copies, meaning that there are almost no truly unique copies to serve as positional landmarks within the NORs. As a consequence, we find it necessary to have sequence reads that span multiple rRNA genes such that, collectively, sets of contiguous genes have sufficient variation to identify unique patterns of rRNA gene variants within a single read. The chaining of these patterns allows for contig assembly.

I have created a computational pipeline, using existing tools and custom scripts, to identify rRNA gene variant types within long reads. These variant calls are then used to identify unique groups of genes within the NOR. Unique sequence 'tags' are then inserted into reads corresponding to these regions, which facilitates contig assembly and sequence polishing. To date, this approach has enabled the assembly of NOR sequence contigs in excess of 1 million basepairs, suggesting that complete, or nearly complete, NOR assemblies may be possible. NOR assemblies are expected to provide insight into nucleolar dominance and allow studies of meiotic recombination (and its suppression) and other chromosomal phenomenon that involve the NORs.

The computational tools being developed are written to be flexible for use with other genomes or other DNA repeats, requiring only that there be sufficient sequence variation within individual repeats to create unique patterns among multiple repeats.

**Unveiling the variability and organization of the ribosomal RNA gene repeats with long sequencing technologies.**

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Transcription of the ribosomal RNA genes (rDNA) by RNA polymerase I (Pol I) within the nucleoli is a critical step in ribosome biogenesis. The rDNA is organized into large arrays of tandem repeats, with on average 400–600 rDNA copies per haploid genome in humans. However, little is known about rDNA variation and structure, and specifically whether sequence and structural variants could affect expression of the rDNA or activity of the assembled ribosomes. Current missing knowledge is mainly due to the rDNA repeat arrays being one of the last remaining gaps in the human genome. We have devised an innovative approach that leverages long read sequencing technologies to investigate the properties of the rDNA repeat arrays. Using sequencing data from the lymphoblastoid cell line (LCL) GM24385, we identified 918 reads of length >100kb containing a total of 3300 candidate rDNA repeat units, averaging 3-4 units per read. The rDNA units had highly conserved sizes, suggesting that they maintain full coding potential. We performed direct RNA sequencing of rRNAs from the cytosolic fraction of the same cell line (LCL) and found that 99 variants found in the rDNA were expressed in rRNAs. We further predicted the patterns of CpG methylation on these reads and found two starkly contrasting methylation patterns with similar proportions (~50% each). One with the rRNA genes and promoter unmethylated and the IGS highly methylated and another pattern with the rRNA genes, the promoter, and the IGS highly methylated. Strikingly, most (~90%) of the 918 reads analysed had the same methylation pattern in all units, rather than alternating between methylated and unmethylated. Moreover, we found reads with inversions resulting in units with diverging and converging transcriptional orientations. The variation in sequence, structure, and methylation we have uncovered represent previously unrecognized ways through which the rDNA may influence ribosome rRNA composition and cell function.

# Session 4, Chair: Christoph Mueller

**Magdalena Boguta** - "Biogenesis of yeast RNA polymerases III involves co-translational assembly mechanism"

**Carlos Fernández-Tornero** - "The role of RNA polymerase I in ribosomal DNA protection against UV light-induced DNA damage"

**Ruth Q. Jacobs** - "Defining the divergent enzymatic properties of the eukaryotic RNA polymerases"

**Akihito Fukudome** - "Structure of RNA-dependent RNA polymerase 2 and its implications for double-stranded RNA synthesis in RNA-directed DNA methylation"

**Biogenesis of yeast RNA polymerases III involves co-translational assembly mechanism**

Małgorzata Cieśla[1], Tomasz Turowski[2,] David Tollervey[2] and Magdalena Boguta[1*]
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The biogenesis of eukaryotic RNA polymerases is poorly understood. By using a combination of yeast genetic and molecular approaches we identified a regulatory link between Rbs1, a Pol III assembly factor, and Rpb10, a small subunit shared by RNA polymerases. Overexpression of Rbs1 increased the abundance of both *RPB10* mRNA and the Rpb10 protein, which correlated with suppression of Pol III assembly defects. The sequence of Rbs1 protein contains two RNA-binding domains, R3H and SUZ, and a large unstructured region with a potential prionogenic domain. The orthologues of Rbs1 are present in other eukaryotes, including humans. We have shown that Rbs1 binds poly(A)mRNA in manner dependent on the R3H domain. Genome-wide RNA binding by Rbs1 was characterized by UV cross-linking based approach. We demonstrated that Rbs1 directly binds to the 3' untranslated regions (3'UTRs) of many mRNAs including transcripts encoding Pol III subunits, Rpb10 and Rpc19. Together with two other subunits, Rpc40 and Rpb12, Rpb10 and Rpc19 are suggested to form the initial Pol III sub-assembly. We propose that this sub-assembly is co-translationally seeded while the Rpb10 subunit is synthesized by cytoplasmic ribosome machinery. The translation of Rpb10 is stimulated by Rbs1 protein, which binds to the 3'UTR of *RPB10* mRNA and hypothetically brings together Rpc19 and Rpc40 subunits to form the αα-like heterodimer (analogue of αα bacterial module). We suggest that such a co-translational mechanism is involved in the assembly of Pol III and Pol I complexes.
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## The role of RNA polymerase I in ribosomal DNA protection against UV light-induced DNA damage

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DNA lesions threaten cell life and must be repaired to maintain genome integrity. During transcription, RNA polymerases actively scan DNA to find lesions and trigger their repair. In growing eukaryotic cells, about 60% of the total transcriptional activity involves the synthesis of ribosomal RNA (rRNA) by RNA polymerase I (Pol I), a 14-subunit macromolecular machine with unique regulatory features [1,2]. Accordingly, transcribing Pol I monitors ribosomal DNA (rDNA) integrity and influences cell survival, but how this enzyme handles DNA lesions remains largely unknown. We used cryo-EM and *in vitro* transcription tests to investigate Pol I transcriptional stalling by one of the most common UV light-induced lesions, i.e. cyclobutane pyrimidine dimers (CPD) [3]. A two-step mechanism operates in Pol I to firmly stall the enzyme at CPD lesions, whereas RNA polymerase II (Pol II), which scans for damage within protein-coding genes, is able to bypass such lesions. First, bridge helix residue Arg1015, which is unique in Pol I, contacts the lesion and significantly reduces the bypass rate, as we confirm by mutational analysis. Second, the intrinsic RNA cleavage activity of Pol I, which in Pol II requires binding of transcription factor IIS (TFIIS), removes RNA nucleotides opposite the lesion. Our results suggest that this dual mechanism strongly blocks rRNA synthesis to hamper incorporation of mutations into ribosomes. These findings open the avenue to unravel the molecular mechanisms underlying cell endurance to lesions on rDNA.

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2. Fernández-Tornero C (2018) Transcription 9:248-254
3. Sanz-Murillo M, Xu J, Belogurov GA, Calvo O, Gil-Carton D, Moreno-Morcillo M, Wang D, Fernández-Tornero C (2018) Proc. Natl. Acad. Sci. USA 115:8972-8977

**Defining the divergent enzymatic properties of the eukaryotic RNA polymerases**

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Eukaryotes have at least three nuclear, DNA-dependent RNA polymerases (Pols) responsible for synthesizing all the genome-encoded RNA required by the cell. Since the discovery of the Pols, their characterization has been executed under disparate experimental conditions, making direct comparisons of the Pols challenging. To that end, we have devised a method whereby the Pols can be directly compared on a millisecond time scale using a chemical-quenched flow instrument and a versatile, promoter-independent, *in vitro* transcription assay. Unlike most techniques used to investigate transcription elongation, our method provides the time resolution necessary to measure rate constants governing the addition of individual nucleotides. Here, we determine key enzymatic properties of *Saccharomyces cerevisiae* Pols and compare them under identical experimental conditions. We find that single and multi-nucleotide addition by Pol I is faster than by Pol II and III. Pol I elongation complexes (ECs) were found to be less stable than both Pol II and III ECs. Additionally, we find that Pol I is the most error prone of the Pols. Taken together, our results reveal key enzymatic differences between Pols I, II, and III that provide new insights into their evolutionary divergence.

Jacobs, R.Q., Ingram, Z.I., Lucius, A.L., and Schneider, D.A. (2020) Defining the divergent enzymatic properties of RNA polymerases I and II. *Journal of Biological Chemistry*, 296, 100051

**Structure of RNA-DEPENDENT RNA POLYMERASE 2 and its implications for double-stranded RNA synthesis in RNA-directed DNA methylation**

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Abstract:

In plants, yeast, fungi and nematodes, RNA-dependent RNA polymerases (RdRP) play critical roles in RNA-mediated gene silencing. In *Arabidopsis thaliana*, RNA-DEPENDENT RNA POLYMERASE 2 (RDR2) physically associates with DNA-dependent NUCLEAR RNA POLYMERASE IV and their activities are tightly coupled to generate double-stranded RNAs (dsRNAs) from DNA templates. Resulting dsRNAs are then processed into short-interfering RNAs that guide RNA-directed DNA methylation and transcriptional gene silencing. Using single-particle cryo-electron microscopy, we determined the structure of RDR2 at 3.1 Å resolution. The catalytic region of RDR2 shares structural similarity with the *Neurospora crassa* RdRP, QDE-1. However, the RDR2 structure also reveals that the N-terminal region, which is missing in the partial QDE-1 structure, includes a RNA-recognition motif (RRM) adjacent to a positively charged channel that extends to the catalytic center. The distance from the RRM to the three aspartates at the active site corresponds to the length of a single-stranded RNA of ~7 nt. Consistent with this observation, we show that RNAs longer than 8 nt serve as efficient templates for RDR2. Likewise, RDR2 can transcribe the RNA strand of RNA-DNA hybrids, but 9 or more nt at the 3' end of the RNA needs to be unpaired in order to be engaged by RDR2. In experiments mimicking the arrangement of DNA and RNA strands following Pol IV transcription, RDR2 will also engage unpaired RNA 3' ends generated upon reannealing of the template and nontemplate DNA strands, again with a requirement for ~10 nt of unpaired RNA. Collectively, our structural and biochemical data suggest a mechanism in which Pol IV arrest and backtracking causes its transcripts' 3' ends to become unpaired from the template DNA, allowing RDR2 to engage the nascent transcripts and extract them from Pol IV as a consequence of dsRNA synthesis.

# Session 5, Chair: Finn Werner

**Rachel McNamar** - "Mammalian PAF49, the ortholog of the nonessential yeast RNA polymerase I subunit RPA34, is essential
for rDNA transcription"

**Christoph Engel** - "Structural basis of RNA polymerase I transcription"

**Bruce A. Knutson** - "Molecular Topology of RNA Polymerase I Upstream Activation Factor"

**Tomasz W. Turowski** - "Functional analysis of RNA polymerase I reveals common features of transcription machinery"

**Mammalian PAF49, the ortholog of the nonessential yeast RNA polymerase I subunit RPA34, is essential for rDNA transcription**

Rachel McNamar[1], Katrina Rothblum[1], and Lawrence I. Rothblum[1]

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The regulation of ribosome biogenesis (RB) plays a central role in maintaining cellular homeostasis and supporting cell growth. The rate-limiting step in this process is transcription of the ribosomal RNA genes by RNA polymerase I (Pol I). Dysregulation of RB can contribute to pathologies such as cancer, cardiac hypertrophy, and ribosomopathies. Many pathways play a role in the regulation of rDNA transcription. Two mammalian rDNA transcription factors that are involved in this regulation are **P**olymerase **A**ssociated **F**actor (PAF53) and PAF49. The purpose of this study is to determine the role(s) the PAFs play in facilitating rDNA transcription and characterize the downstream physiological effects of directly inhibiting their functions.

Previously, our lab used CRISPR/Cas9 in conjunction with an auxin inducible degron to target and rapidly knock down (KD) PAF53. We have used this same system to perform mirror studies in HEK293 cells that have been engineered to inducibly KD PAF49. Our preliminary data show that PAF49 is also required for both rDNA transcription and cell proliferation. These results contradict the studies performed in yeast that demonstrate that the yeast ortholog Rpa34 is a nonessential protein. Further, our lab has found that PAF53 and PAF49 are co-regulated posttranslationally. When PAF49 is knocked down, PAF53 is also degraded. Dimerization of PAF49 with PAF53 is not sufficient to stabilize PAF53. The addition of the polymerase binding domain of PAF49 to its dimerization domain is sufficient to stabilize PAF53 and facilitate rDNA transcription. These findings are significant because they aid in further understanding the process of rDNA transcription by Pol I and the physiological consequences of inhibiting this process, *i.e.* nucleolar stress and cell arrest and/ or death.

**Structural basis of RNA polymerase I transcription**

Michael Pilsl, Florian B. Heiss, Julia L. Daiß, Philipp Becker, Mona Höcherl, and Christoph Engel*

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Synthesis of ribosomal RNA (rRNA) requires RNA polymerase (Pol) I in all eukaryotes. Structural and functional characterization of Pol I has revealed unexpected similarities and striking differences compared to other transcription systems. In a high-resolution single-particle cryo-Electron Microscopy (cryo-EM) reconstruction, we trapped an early initiation intermediate structure comprising initiation factors Rrn3 and heterotrimeric core factor (CF). Comparative modelling revealed that efficient promoter-backbone interaction is achieved by combined re-arrangements of flexible regions in CF subunits Rrn7 and Rrn11 that are required to achieve initiation *in vitro*. These regions are not present in TFIIB, a general Pol II initiation factor and counterpart to Rrn7. Furthermore, structure-function analysis illustrates how destabilization of the melted DNA region correlates with contraction of the polymerase cleft upon transcription activation, thereby combining promoter recruitment with DNA-melting.

Independently, we studied whether regulatory mechanisms specific to Pol I are conserved among organisms. Three cryo-EM reconstructions of Pol I from fission yeast *Schizosaccharomyces pombe* in different functional states indicate that 'activation by cleft contraction' and 'hibernation by dimerization' are employed across species. Strikingly, dimerization is achieved independent of the 'connector' domain but relies on two interfaces structurally divergent from *S. cerevisiae*.

Our analyses highlight the divergent nature of Pol I transcription systems from their counterparts and suggest conservation of regulatory mechanisms among organisms.

Michael Pilsl, and Christoph Engel. Structural basis of RNA polymerase I pre-initiation complex formation and promoter melting. Nature Communications 11, 1206 (2020)

Florian B. Heiss, Julia L. Daiß, Philipp Becker, and Christoph Engel. Conserved strategies of RNA polymerase I hibernation and activation. Nature Communications 12, 758 (2021)

**Molecular Topology of RNA Polymerase I Upstream Activation Factor**

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Upstream Activation Factor (UAF) is a multifunctional transcription factor in *Saccharomyces cerevisiae* that plays dual roles in activating RNA Polymerase I (Pol I) transcription and repression of Pol II. For Pol I, UAF binds to a specific upstream element in the rDNA promoter and interacts with two other Pol I initiation factors, the TATA-Binding Protein (TBP) and Core Factor (CF). We used an integrated combination of chemical crosslinking mass spectrometry (CXMS), molecular genetics, protein biochemistry, and structural modeling to understand the topological framework responsible for UAF complex formation. Here, we report the molecular topology of the UAF complex, describe new structural and functional domains that play roles in UAF complex integrity, assembly, and biological function, and provides roles for previously identified UAF domains that include the Rrn5 SANT and Histone Fold domains. We highlight the role of new domains in Uaf30 that include an N-terminal Winged Helix domain and a disordered Tethering domain as well as a BORCS6-like domain found in Rrn9. Together, our results reveal a unique network of topological features that coalesce around a histone tetramer-like core to form the dual functioning UAF complex.

Knutson BA, Smith ML, Belkevich AE, Fakhouri AM. Molecular Topology of RNA Polymerase I Upstream Activation Factor. Mol Cell Biol. 2020 Jun 15;40(13):e00056-20.

**Functional analysis of RNA polymerase I reveals common features of transcription machinery**

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To characterize basic transcriptional mechanisms, we analyzed elongation and backtracking by RNA polymerase I in *S.cerevisiae*. Analysis of Miller chromatin spreads and mapping RNAPI using UV crosslinking, revealed a marked 5' bias and strikingly uneven local polymerase occupancy, with an apparently regular distribution of peaks and troughs, particularly over the 5’ region of the pre-rRNA. Two features of the nascent transcript correlated with RNAPI distribution: strong structures close to the polymerase promote forward translocation and limit backtracking, as confirmed by *in vitro* experiments, whereas high G+C-content within the transcription bubble slows elongation. We developed a mathematical model for RNAPI, combining effects of DNA torsion under transcriptional stress with RNA folding energy. This links *in vitro* measurements with RNAPI profiling and, among other conclusions, indicates that cotranscriptional folding indeed reduces backtracking. Analyses of the *in vivo* distributions of RNAPII and RNAPIII indicate that these are also sensitive to nascent transcript folding. We are now applying related approaches to understand transcription termination and other systems.

Turowski TW, Petfalski E, Goddard BD, French SL, Helwak A, Tollervey D. Nascent transcript folding plays a major role in determining RNA polymerase elongation rates. *Molecular Cell* 79(3)488-503, 2020

# Session 6, Chair: Bruce Knutson

**Nisreen Chahid** - "Novel strategies for improving Pichia pastoris as an expression platform"

**Sebastian Kruse** - "Purification of native chromatin templates for investigation of RNA polymerase I promoter-specific transcription in vitro"

**Laura Martins** - "Transcriptional regulation of the four putative chromatin remodeling factors, CLSY1-4, in Arabidopsis thaliana"

**V. Miguel Palomar** - "The plastid chromatin organization is determined by protein binding"

**Katrin Schwank** - "Reconstitution of RNA polymerase I to investigate the function of the lobe binding subunits in in vitro assays"

**Rebecca Sizer** - "Using tRNA genes to improve the yield of therapeutic antibodies"

**Kanwal Tariq** - "Noncoding RNAs from human rDNA spacer regulate chromatin organization and accessibility"

**Thejaani Udumanne** - "Characterising changes in rDNA chromatin during malignant transformation"

**Guanghui Xu** - "Identifying novel factors that alter DNA methylation patterns in Arabidopsis"

**Novel Strategies for improving Pichia pastoris as an expression platform**

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*Pichia pastoris* (aka *Komagataella pastoris* and *Komagataella phaffi*) is becoming increasingly popular in the industrial biotechnology sector for its ability to produce and secrete gram amounts of recombinant protein per litre of culture. However, compromised expression levels have been observed in isolates of the same transgene-expressing strains, suggesting that epigenetic silencing may be playing a role in limiting expression in *Pichia pastoris* systems. In related yeast species *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, tDNAs have been identified which act as barriers to block heterochromatic silencing. We have used an assay based on the *Saccharomyces cerevisiae* mating locus to demonstrate that *Pichia pastoris* tDNAs can also block heterochromatic silencing. We plan to test whether these tDNAs can promote the sustained expression of transgenes in *Pichia pastoris*.

**Purification of native chromatin templates for investigation of RNA polymerase I promoter-specific transcription *in vitro***

Sebastian Kruse*, Katrin Schwank, Christopher Schächner, Kristin Hergert, Catharina Schmid, Tobias Fremter, Philipp Milkereit, Herbert Tschochner and Joachim Griesenbeck

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RNA polymerase I (Pol I) transcribes ribosomal (r)RNA genes at high rate in all eukaryotes accounting for more than 60% of total cellular transcription in the model organism *S. cerevisiae* (hereafter called yeast). As all nuclear RNA polymerases, Pol I has to deal with a chromatin template, mainly composed of nucleosomes. There is evidence that rRNA genes become depleted of nucleosomes in the course of Pol I transcription. The basis of the high transcriptional activity of Pol I *in vivo* as well as the process of nucleosome depletion at chromosomal rRNA gene loci are not well understood. Most of our detailed knowledge about the mechanism of Pol I transcription relies on *in vitro* analyses using highly purified Pol I enzymes and components of the Pol I transcription machinery, as well as defined artificial nucleic acid templates. To better reflect the *in vivo* situation, *in vitro* reconstituted nucleosomal transcription templates have frequently been employed in the past. However, it remains unclear in how far those templates reflect the native chromatin state. We have established a method for the purification of native chromatin segments from an episome encompassing a Pol I dependent minigene from yeast. The minigene contains all known functional genetic elements responsible for Pol I transcriptional regulation. Pol I transcription from these minigenes *in vivo* as judged by its chromatin composition and establishment of the nucleosome-depleted chromatin state is indistinguishable from Pol I transcription of chromosomal rRNA genes (see short talk by Christopher Schächner). We will present details on the method used for chromatin isolation and discuss its potential use to reconstitute Pol I transcription in the native chromatin context in defined *in vitro* conditions (see talk by Herbert Tschochner & Joachim Griesenbeck).

**Transcriptional regulation of the four putative chromatin remodeling factors, CLSY1-4, in *Arabidopsis thaliana***

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To maintain genome integrity and regulate gene expression, eukaryotic organisms modify their DNA and histones via the addition of epigenetic modifications. In Arabidopsis, DNA methylation in all sequence contexts is established by the RNA-directed DNA methylation (RdDM) pathway, which requires two plant specific RNA polymerases. Pol-IV is required to generate 24-nucleotide (nt) small interfering RNAs (siRNAs) and Pol-V is required to generate long non-coding RNAs, both of which are essential for the targeting of DNA methylation. Previous studies led to the identification of several factors that co-purify with the Pol-IV complex, including four putative chromatin remodeling factors, CLASSY1-4 (CLSY). Initial characterization of the CLSYs family revealed that they control 24nt-siRNA production and DNA methylation patterns by facilitating the targeting of Pol-IV in a locus-specific manner. Moreover, our recent work shows that the CLSY family also controls tissue-specific DNA methylation in concordance with their distinct expression patterns at specific developmental stages. Despite recent progress in the field, the mechanisms controlling the expression patterns of epigenetic regulators, including the *CLSY* genes, are poorly understood. To understand how the *CLSY* genes are regulated, we are combining high throughput screening and bioinformatics approaches to identify transcription factors (TFs) that bind their promoters. To validate our best hits *in vivo*, *CLSY* expression profiles and siRNA levels we be assessed in mutant backgrounds to reveal TFs with roles in RdDM. These approaches will identify the transcriptional networks that control the tissue-specific expression patterns of the *CLSYs* and enable the generation of diverse methylation patterns during plant development.

**The plastid chromatin organization is determined by protein binding**

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Although the organization of the nuclear chromatin and its role in transcriptional regulation are well defined mechanisms, little is known about the structural organization of the plastid nucleoid and its consequences in the plastid gene regulation. In this work, using several high throughput-sequencing techniques, we found that the plastid nucleoid has a complex and specific organization, which is primarily caused by protein binding, transcription and association with membranes. Moreover, these features are variable among different regions in the plastid genome and are dynamically modulated during the chloroplast development. Given those evidences, we propose that the plastid nucleoid structure is complex, stable and functionally relevant.

**Reconstitution of RNA polymerase I to investigate the function of the lobe binding subunits in *in vitro* assays**

Katrin Schwank*, Sebastian Kruse, Catharina Schmid, Kristin Hergert, Tobias Fremter, Philipp Milkereit, Joachim Griesenbeck and Herbert Tschochner

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The eukaryotic RNA polymerase I (Pol I) is a highly specialized and complex multisubunit enzyme that transcribes the ribosomal RNA gene. In yeast, the Pol I specific subunits Rpa34.5/Rpa49 and Rpa12.2 bind to the lobe structure of Pol I and are suggested to have an impact on transcription initiation, elongation and passage through nucleosomes. To study the function of different subdomains of these subunits in transcription elongation, we use a purified Pol I Δ12 enzyme lacking the lobe binding subunits. Furthermore, different N- and C-terminally truncated protein variants of Rpa34.5/Rpa49 and Rpa12 were purified from *E.coli*. Afterwards, the recombinantly expressed proteins were used for assembly with the Pol I Δ12 enzyme. Then, the reconstituted Pol I mutants can be tested in different *in vitro* assays to investigate the interplay of distinct domains of the lobe binding subunits and their function in transcription elongation. In this talk, we would like to present our reconstitution system and one example for its application.

**Using tRNA genes to improve the yield of therapeutic antibodies**

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Therapeutic antibodies are essential for the treatment of many devastating illnesses, including cancer. For example, trastuzumab, one of the first therapeutic antibodies approved, can nearly double the life expectancy of an individual suffering from a subtype of breast cancer (1). However, these benefits come with a cost, something that has restricted the use of trastuzumab to the USA and Western Europe. One way to drive down the cost of therapeutic antibodies is to improve yield production. The yield is highly dependent on the site of integration of the transgene into the genome; integration into condensed heterochromatin leads to silencing/very low yield. Even the integration into euchromatin can be unstable, as heterochromatin can spread over the transgene and silence it. Over the past few years, research into the use of insulator elements to protect transgenes from heterochromatic silencing has evolved. Barrier insulator elements are DNA sequences that shield nearby euchromatic regions from heterochromatic spread. Interestingly, certain tRNA genes have been shown to possess barrier activity, yet this activity is not a characteristic of all tRNA genes (2–4). My work aims to characterize how tRNA genes can act as barriers, with the overall goal of identifying the best tRNA gene barriers for industry. To do this, I will be using a combination of techniques including chromatin immunoprecipitation, cloning, flow cytometry and bioinformatics. Improving the current understanding of how tRNA genes act as barriers will hopefully drive down the cost of production of therapeutic antibodies and increase their accessibility.

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**Noncoding RNAs from human rDNA spacer regulate chromatin organization and accessibility**

Stefanie Böhm, Kanwal Tariq*, Jaclyn Quin, Judit Domingo Prim, Sonja Peter, Anna Vintermist, Antoni Ganez Zapater, Neus Visa, and Ann-Kristin Östlund Farrants

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Human rDNA exists in multiple genetic and epigenetic states. This allows cells to regulate rRNA transcription as well as maintain the nucleolar architecture for diverse functions. Noncoding RNAs arising from the rDNA repeats, such as pRNA and PAPAS, have been previously reported to regulate 45Spre-rRNA transcription by coordinating with chromatin remodelling complexes. In the present study, we establish the function of two novel IGS lncRNAs; IGS32as and IGS38s, in regulating rDNA repeats. Our data demonstrates that both lncRNAs are required for maintaining the rRNA levels in the cells. Noncoding RNA IGS32as, a PolI transcript, is necessary for recruitment of HP1α to establish heterochromatin. In contrast, IGS38s, a PolII transcript, maintains an accessible chromatin conformation at the rDNA promoter while keeping the upstream spacer promoter comparatively inaccessible. This allows the RNA Pol1 machinery to proceed with the rRNA transcription.

**Characterising changes in rDNA chromatin during malignant transformation**

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Ribosomal RNA (rRNA) gene (rDNA) transcription by RNA Polymerase I (Pol-I) occurs in the nucleolus, the site of ribosome biogenesis, and determines proliferation and cell growth in all organisms. There are ~400 copies of rDNA genes per diploid genome, however remarkably less than 50% are typically transcribed in normal cells. It is well documented that elevated Pol-I transcription is a common feature in malignancy, and that Pol-I inhibition prolongs the overall survival of lymphoma-bearing mice. To take full advantage of targeting Pol-I transcription as a cancer therapy, a detailed understanding of rDNA chromatin structure, the transcription factors involved, and how they change during malignant transformation is crucial.

This study utilises a mouse model of spontaneous B-cell lymphoma (Eμ-*Myc*) to characterise the epigenetic and transcriptional changes in malignancy. Chromatin Immunoprecipitation Sequencing (ChIP-seq) revealed that the transition from wildtype to pre-malignant to malignant B cells is associated with marked changes in rDNA chromatin. This includes increased binding of Pol-I and upstream binding factor (UBF), and depletion of linker histone H1 and repressive histone marks at regulatory and coding regions. Robust quantification of specific binding/enrichment across rDNA was facilitated by novel bioinformatics approaches that accommodate the highly repetitive nature of rDNA locus. We are currently evaluating how rDNA epigenetic state may also affect the global transcriptional activity via interacting genomic regions, and furthermore the potential rDNA copy number variability observed in malignancy. These findings will provide further insight into the role of rDNA chromatin in malignant transformation and its potential as a therapeutic target.

**Identifying novel factors that alter DNA methylation patterns in *Arabidopsis***

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DNA methylation is an important epigenetic mark that regulates gene expression and silences transposable elements to ensure genome stability. In both plants and animals, DNA methylation is targeted by small RNAs, however, unlike in animals where Pol II is required for small RNA production, in plants two additional polymerases, Pol IV and Pol V, evolved to serve this function. These polymerases generate 24-nt siRNAs and long non-coding RNAs, respectively, to direct DNA methylation to specific genomic targets as part of the RNA-directed DNA methylation (RdDM) pathway. We previously showed that four chromatin remodeling factors (CLSY1 - CLSY4) regulate DNA methylation patterns by controlling the targeting of Pol-IV and thus, the production of 24-nt siRNAs. Interestingly, we found that CLSY1 and CLSY2 are mainly targeted to gene-rich regions in the chromosome arms, while CLSY3 and CLSY4 are mainly targeted to pericentromeric heterochromatin. For CLSY1 and CLSY2 this targeting requires SHH1, an H3K9me reader, but for CLSY3 and CLSY4 the mechanism of targeting remains poorly understood. Thus, to gain insights into how CLSY3 and CLSY4 regulate DNA methylation patterns, we initiated a forward genetic screen. For this screen, we developed a PCR-based methyl-cutting assay to identify reduced methylation levels specifically at sites regulated by CLSY3 and CLSY4 using an Ethyl Methyl Sulfonate (EMS) mutagenized collection of Arabidopsis plants. Five mutant lines that mimic the *clsy3* mutant in the methyl-cutting assay were identified. PCR-based sequencing confirmed that the mutants do not harbor a mutation in *CLSY3*, demonstrating they are likely novel factors important for DNA methylation. For the underlying genes, we expect to identify several types of players, such as chromatin readers, chromatin state modifiers, transcription factors, etc. The identification and characterization of these genes hold the potential to fill the gaps in understanding how Pol IV targeting to both open and closed chromatin is facilitated by the CLSYs.

# Session 7, Chair: Ale Vannini

**Michael Bartlett** - "Positioning of archaeal general transcription factors TFB and TFE during transcription initiation"

**Ewan Ramsay** - "Structural characterisation of human RNA polymerase III"

**Agata Misiaszek** - "Cryo-EM structures of human RNA polymerase I"

**Małgorzata Cieśla** - "The R3H domain-containing Rbs1 protein and the Upf1 helicase modulate the expression of Rpb10, a small subunit common to RNA polymerases"

**Gwenny Cackett** - "African Swine Fever Virus – from transcriptome to mechanism"

**Positioning of archaeal general transcription factors TFB and TFE during transcription initiation**

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Transcription initiation by archaeal RNA polymerase is facilitated by the general transcription factors TFB and TFE alpha, homologs of eukaryotic TFIIB and TFIIE alpha. Events in early initiation, including open complex formation and maintenance, RNA-DNA duplex placement, and RNAP rearrangements are likely affected by TFB and TFE, but the details of how TFB and TFE interact with RNAP and promoter DNA during these events are incomplete. To address this, mutant TFB and TFE proteins were made containing the photoreactive amino acid *p*-benzoyl phenylalanine (Bpa) at specific positions. Bpa-containing mutant proteins were used to form pre-initiation complexes, as well as initiated complexes containing short transcripts. Cross-links were induced by UV irradiation, and cross-linked positions in DNA were mapped by DNA label transfer or primer extension. The results provide amino acid to nucleotide resolution maps of protein DNA contacts for these transcription factors, and indicate changes in protein-DNA interactions that occur as transcription initiation proceeds.

**Structural characterisation of human RNA polymerase III**

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RNA polymerase III (Pol III) is a specialised polymerase, concerned with the production of structured, non-translated RNAs such as the entire pool of tRNAs [1]. Pol III is highly regulated in a cell cycle and cell-type specific manner, with loss of this regulation associated with cancer, neurodegenerative disease, developmental disorders and loss of viral immunity [2,3]. Extensive structural and biochemical studies using the *S. cerevisiae* system have considerably increased our understanding of Pol III structure and transcription. Despite this, our understanding of the homologous human machinery is still not complete. Notably, the human enzyme incorporates an additional large C-terminal extension in the regulatory subunit RPC5, for which a function is still not described [4]. Therefore, our study sought to determine the structure of the human Pol III enzyme and in particular the extension in RPC5. Tagged, endogenous human Pol III was isolated from CRISPR-modified HeLa cells and subject to analysis using Cryo-EM. This structural information was integrated with X-ray crystallography structures and SAXS modelling of the RPC5 C-terminal extension to provide one of the first descriptions of human Pol III structure [5]. Exploiting this, we were able to map causative mutations in various Pol III-associated neurodegenerative and developmental conditions, rationalising their effects on the human Pol III assembly. Going further, functional assessment in cells identified a role for the RPC5 C-terminal extension in stability maintenance of the RPC5 subunit and of the entire polymerase [5]. Taken together, this represents one of the first characterisations of the human Pol III enzyme and provides a starting point for further investigation of the human Pol III machinery.

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**Cryo-EM structures of human RNA polymerase I**

Agata D. Misiaszek[1,*], Mathias Girbig[1], Helga Grötsch[1], Siddarth Narasimhan[1], Brice Murciano[1], Aleix Lafita[2], Florence Baudin[1], Alex Bateman[2], Christoph W. Müller[1]

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RNA polymerase I (Pol I) specifically synthesizes ribosomal RNA. Upregulation of Pol I activity is linked to cancer, while mutations in the Pol I machinery lead to developmental disorders. Here, we report the cryo-EM structure of human Pol I in its elongating state at 2.7 Å resolution. Together with the recent human Pol III structure[1], it completes the gallery of human RNA polymerases. We observe a double-stranded conformation of RNA in the exit tunnel that may support Pol I processivity. Our structure confirms that human Pol I consists of 13 subunits with only one subunit forming the Pol I stalk. Additionally, the structure of human Pol I in complex with the initiation factor RRN3 at 3.1 Å resolution reveals stalk flipping upon RRN3 binding. We also observe an inactivated state of human Pol I bound to an open DNA scaffold at 3.2 Å resolution. Lastly, the high-resolution structure of human Pol I allows mapping of disease-related mutations and may aid in the design of Pol I-specific inhibitors.

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**The R3H domain-containing Rbs1 protein and the Upf1 helicase modulate the expression of Rpb10, a small subunit common to RNA polymerases**

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Previous work supported a role of Rpb10, a small subunit that is common to three RNA polymerases, in assembly of yeast Pol I and Pol III (1, 2). We used a combination of genetic and molecular approaches to explore the assembly of Pol III complex. We identified a regulatory link between Rpb10 and Rbs1 protein previously identified in our laboratory as a Pol III assembly factor (3). Overexpression of Rbs1 increased the abundance of both *RPB10* mRNA and the Rpb10 protein, which correlated with suppression of Pol III assembly defects in the yeast mutant *rpc128-1007*. We have shown that Rbs1 is a poly(A)mRNA-binding protein and that R3H domain is required for Rbs1-mRNA interactions. Mutational analysis identified R3H domain in Rbs1 and 3'UTR region in *RPB10* mRNA necessary for genetic enhancement of Pol III biogenesis. Rbs1 also binds to Upf1 helicase, a key component in nonsense-mediated mRNA decay (NMD) and levels of *RPB10* mRNA were increased in a *upf1*Δ strain. We propose that Rbs1 functions by opposing mRNA degradation, at least in part mediated by NMD pathway.

This work was supported by the National Science Centre [UMO-2017/25/B/NZ1/01889].

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**African Swine Fever Virus – from transcriptome to mechanism**

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African Swine Fever Virus (ASFV) is a nucleocytoplasmic large dsDNA virus that causes lethal and incurable haemorrhagic fever in pigs. ASFV carries out RNA transcription and modification independently of host cell machinery, encoding an 8-subunit RNA polymerase (RNAP), a poly-A polymerase, and mRNA capping enzyme. Strikingly, the ASFV transcription system is reminiscent of the Pol II-like system: including homologs of TBP and TFIIB for transcription initiation, with additional Vaccinia virus-like viral transcription factors including D6/A7 [1].

We have brought an integrated functional genomics approach to bear on the ASFV transcription system to define its qualitative and quantitative features [2]. We have applied a combination of NGS techniques including CAGE-seq, RNA-seq and 3' RNA-seq to determine (i) the global transcription start site (TSS) map, (ii) transcription termination site (TTS) map, and (iii) the transcriptome of ASFV-BA71V strain. We have furthermore characterised the temporal transcription programme of the virus, analysing differential gene expression using RNA isolated from infected *Vero* cells at 5- and 16-hours post-infection, which identified 91 of 151 genes as differentially expressed. We assigned primary TSSs of 151 (from 153 total) ASFV genes with high confidence, which enabled the rigorous functional annotation of the virus genome, including the discovery of novel genes. Alignments and MEME motif searches of DNA sequences upstream of TSSs revealed a distinct early (EPM) and late (LPM) gene promoter motifs. The EPM consensus signature and its location relative to the TSS is similar to promoters of early Poxvirus genes; the EPM is likely recognised by the virus-specific heterodimeric D6-A7 initiation factor, including a predicted ATP-dependent helicase. We have produced recombinant D6-A7 in insect cells, obtained preliminary structural information by negative staining EM, and investigated the interaction of D6-A7 with early ASFV promoter templates using EMSAs. This work has given novel and extensive insight into ASFV gene transcription and the first of its kind on this global genomic scale.

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# Session 8, Chair: Rich Maraia

**Dina Grohmann** - "DNA origami-based single-molecule force spectroscopy elucidates RNA Polymerase III pre-initiation complex stability

**Francisco Gutiérrez-Santiago** - "Bud27 and its role in RNA pol III transcription"

**Nayef Jarrous** - "Coordination of transcription and processing of tRNA in human cells"

**Tom Moss** - "Mechanisms of rDNA promoter recognition and transcription initiation underly the UBTF neuroregression syndrome and the action of CX-5461"

**DNA origami-based single-molecule force spectroscopy elucidates RNA Polymerase III pre-initiation complex stability**

Kevin Kramm, Tim Schröder, Jerome Gouge, Kapil Gupta, Imre Berger, Alessandro Vannini, Philip Tinnefeld and Dina Grohmann

Important regulatory events in the cell occur at the level of transcription initiation to achieve differential gene expression. In the archaeal-eukaryotic transcription systems, the general transcription initiation factors TBP (TATA-binding protein) and TF(II)B (transcription factor B) are highly conserved. However, additional non-homologous factors are required for initiation in the eukaryotic RNAP I, II and III system. The reason for the emergence and strict requirement of the additional initiation factor Bdp1 in the RNA polymerase (RNAP) III system, for example, remained elusive. Over the last years, we integreated biochemical with single-molecule approaches to gain mechanistic and quantitative insights into the dynamics of TBP-dependent DNA bending in the archael and eukaryotic transcription system. A poorly studied aspect in this context is the effect of DNA strain arising from DNA compaction and transcriptional activity on initiation complex formation. We a made use of a newly developed DNA origami-based force clamp to follow the assembly of archaeal and human initiation complexes in the RNAP II and RNAP III systems at the single-molecule level under piconewton forces. We demonstrate that TBP-DNA complexes are force-sensitive and TFB/TFIIB is sufficient to stabilise TBP on a strained promoter. In contrast, Bdp1 is the pivotal component that ensures stable anchoring of initiation factors, and thus the polymerase itself, in the RNAP III system. Thereby, we offer an explanation for the crucial role of Bdp1 for the high transcriptional output of RNAP III.

## Bud27 and its role in RNA pol III transcription

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Bud27 is a member of the prefoldin-like family of ATP independent molecular chaperones. Bud27 interacts with Rpb5, a common subunit of the three eukaryotic RNA polymerases. Our group has demonstrated that Bud27 mediates the biogenesis of the three RNA polymerases in an Rpb5 dependent-manner in *Saccharomyces cerevisiae*. Also, a tripartite association between Bud27-Rpb5-RSC might influence transcription elongation mediated by RNA pol II. Moreover, we have recently reported that Bud27 influences the transcription of ribosomal components and ribosome biogenesis in *S. cerevisiae* by modulating the activity of all three RNA polymerases in yeast. Our recent work, in agreement with other authors, shows that the absence of Bud27 impacts the occupancy of RNA pol III on the three types of genes transcribed by this RNA polymerase, the RNA synthesis and transcript maturation. Neither repression by Maf1 nor the phosphorylation status of Rpc53 provides a satisfactory explanation for the observed transcriptional defects of RNA pol III. As described for RNA pol II transcription, Bud27 could influence the activity of RNA pol III by affecting the chromatin status. Thus, we are currently characterizing the composition of the chromatin proteome associated with RNA pol III in the presence and absence of Bud27 by quantitative mass spectrometry.

**Coordination of transcription and processing of tRNA in human cells**

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Maturation of tRNA involves several cleavage events that include the removal of the 5' leader, 3' trailer and short intron from precursor form. These cleavages are executed by RNase P, RNase Z and tRNA splicing complex, respectively. Whereas the structure and function of these enzymes are well-studied as individual entities, the context in which they execute and coordinate their activities remains essentially unknown. New preliminary results show that purified initiation complexes assembled on human intron-containing tRNA genes carry out transcription and processing of precursor tRNAs to mature forms. Size exclusion chromatography reveals that these type II initiation complexes have high molecular weights of ~ $2 \times 10^6$, large enough to amass the polymerase, core transcription factors and relevant processing enzymes. Indeed, mass spectrometry analyses confirm the coexistence of protein subunits of Pol III, TFIIIB, TFIIIC, RNase P, RNase Z and tRNA splicing complex and La antigen in these complexes. Supported by functional studies, we propose that transcription and processing of precursor tRNA are coordinated by assembly of multi-functional initiation complexes. Hence, the fate of tRNA is predetermined before initiation of transcription in human cells.

## Mechanisms of rDNA promoter recognition and transcription initiation underly the UBTF neuroregression syndrome and the action of CX-5461.

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High-resolution ChIP-Seq (DChIP-Seq) has in recent years established a broad understanding of the chromatin structure of the ribosomal RNA genes (rDNA). The data show that the active rDNA form a long nucleosome-free region (NFR) that is maintained by transcription and the HMGbox non-sequence specific DNA binding factor UBTF (UBF). However, UBTF has also been identified as a basal transcription factor for RNA polymerase I (RPI/Pol1/PolR1). Two splice variants of UBTF exist in most mammalian cells, only one of which appears to be essential for gene activity. Mapping of these two variants across the rDNA revealed identical binding profiles throughout the NFR but showed specific differences at the RPI promoters, where only the longer UBTF1 variant was found. Strikingly, the presence of UBTF at the promoters depended on the TBP-TAFI complex SL1 and was lost on deletion of the Taf1b gene. The data showed that preinitiation complex formation on the rDNA is driven by an essential and cooperative interdependence between UBF1 and SL1 and suggested an induced-fit model of promoter recognition. We further showed that the recurrent UBTF-E210K mutation causing pediatric neuroregression limits RPI transcription by affecting UBTF-SL1 cooperation.

Some time ago we noted distinct differences in the mode of RPI transcription initiation between stem cells and differentiated cells and found that the drug CX-5461 partly emulated these differences. CX-5461 is a potential inhibitor of ribosomal RNA synthesis and promising chemotherapeutic agent. We found that while CX-5461 had no effect on transcription elongation, it blocked RPI transcription initiation within seconds of its application and did so by preventing promoter release of the RPI-Rrn3 complex. Unexpectedly, the resulting transcriptional arrest was irreversible and led to an unproductive mode RPI recruitment that correlated with nucleolar stress, inhibition of DNA replication, genome-wide DNA damage and cellular senescence. We believe that CX-5461 targets an as yet unrecognized step in RPI promotion that is normally used to regulate transcription initiation. CX-5461 therefore provides a novel tool in the study of transcription initiation and regulation on the rDNA.

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# Session 9, Chair: Tom Santangelo

**Mathias Girbig** - "Structural basis of RNA polymerase III transcription termination"

**Juanjuan Xie** - "Intrinsic and extrinsic mechanisms cooperate to ensure efficient termination of RNAPIII transcription"

**Saurabh Mishra** - "N terminal-and-Linker domain of the C11 subunit of RNA Polymerase III is necessary and sufficient for termination-associated reinitiation-recycling via interaction with
C37/53 heterodimer"

**Tom Santangelo** - "FttA is a CPSF73 homologue that terminates transcription in Archaea"

**Structural basis of RNA polymerase III transcription termination**

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RNA polymerase (Pol) III is highly specialized for the synthesis of short transcripts such as tRNAs. A crucial specialization is the efficient transcription termination of Pol III at oligo thymine stretches on the non-template (NT) strand of the DNA, enabling effective Pol III recycling. Pol III termination is driven by an interplay between DNA, RNA, the Pol III active site and the subunits C53/C37 and C11 and implies the formation of a pre-termination complex (PTC)[1], but the molecular basis underlying this process is not known. Here, we report cryo-EM structures of the yeast Pol III PTC with and without nucleotides at 2.7 and 2.8 Å resolution, respectively. The NT-strand becomes ordered upon PTC formation and induces contraction of the polymerase core. The fork hairpin II forms a hydrophobic cavity that accommodates the thymine methyl groups. Multiple hydrogen bonds form between the thymine bases and a set of conserved residues in subunit C128. Mutating some of these residues induces termination read-through in *in vitro* transcription assays and leads to growth defects at restrictive temperatures. Direct engagement between C53/C37 and the NT-strand could not be observed, but a Pol III deletion construct, lacking C53/C37 and C11, fails to stabilize the NT-strand as shown by cryo-EM. We propose the following model for Pol III termination: Aided by the presence of C53/C37, C128 recognizes the termination signal, thereby, leads to pausing of Pol III and traps it in a non-productive state. This may favor the insertion of RNA-cleaving C11 into the Pol III active site, which induces opening of the clamp domain, destabilizes the engagement of the Pol core with the DNA:RNA hybrid, and ultimately, releases the RNA and, potentially, assists facilitated recycling.

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**Intrinsic and extrinsic mechanisms cooperate to ensure efficient termination of RNAPIII transcription**

Juanjuan Xie[1,*], Umberto Aiello[1], Nouhou Haidara[1], Yves Clement[1], Mathias Girbig[2], Jana Schmitzova[3], Vlad Pena[4], Christoph Müller[2], Domenico Libri[1] and Odil Porrua[1]
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Transcription needs to be terminated precisely and efficiently to avoid the interference between neighbor genes. Most eukaryotic organisms possess three different RNA polymerases (RNAP). Among them, RNAPIII transcribes of tRNA genes and other short non-coding genes. Unlike RNAPII, that strictly requires extrinsic factors to terminate transcription, the current model posits that RNAPIII termination relies solely on a particular DNA sequence, similarly to bacterial intrinsic termination. RNAPIII terminators are typically stretches of Ts that are presumably sufficient for both RNAPIII pausing and its release from the DNA. In this study we unveil an interaction between RNAPIII and a well-characterized transcription termination factor for RNAPII-dependent non-coding genes, the helicase Sen1. We combine genome-wide high-resolution mapping of transcribing RNAPIII with highly-purified *in vitro* transcription termination assays to analyse the interplay between nucleic acid elements and the trans-acting factor Sen1 in termination of RNAPIII transcription. We find that an important fraction of RNAPIIIs normally read through the primary terminator (i.e. the first T-tract downstream of the 3' end of genes) and terminate at downstream secondary terminators. Importantly, preventing the interaction of Sen1 with RNAPIII decreases the efficiency of termination, indicating a global role for Sen1 in promoting RNAPIII termination. We show that T-tracts are only partially efficient in promoting the release of paused RNAPIII from the DNA and that Sen1 can stimulate RNAPIII release by translocating along the nascent transcript. We provide evidence that Sen1 functions mainly at regions with secondary terminators. Conversely, we find that hairpin-like structures that typically form at RNAPIII transcripts near the primary terminator can also stimulate RNAPIII release.
Taken together, our results redefine the rules that govern efficient termination of RNAPIII transcription.

**N terminal-and-Linker domain of the C11 subunit of RNA Polymerase III is necessary and sufficient for termination-associated reinitiation-recycling via interaction with C37/53 heterodimer**

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RNA polymerase (pol) III achieves high level tRNA synthesis by a unique termination-associated reinitiation-recycling process that involves the termination reinitiation subcomplex C37/53/C11. The C11-CTD is homologous to TFIIS-CTD, which induces the intrinsic RNA 3'-cleavage activity of pol II. And the C11-NTD is homologous to Rpb9, a subunit of pol II. In addition to RNA 3'-cleavage activity, C11 is also known to participate in transcription reinitiation-recycling in conjunction with the heterodimeric subunits, C37/53 which are related to TFIIFa/b and A49/34.5 heterodimers associated with pol II and pol I, respectively. However, while the role of C37/C53 in transcription has been characterized in detail, understanding of a mechanism for C11 involvement in the pol III transcription cycle has remained obscure.

Here, we show that the isolated C11-CTD can induce the intrinsic RNA 3'-cleavage activity of pol III, and the isolated C11-NTD stimulates Pol III termination by C37/53 but not reinitiation-recycling which requires the NTD-linker (NTD-L). By an approach different from what led to the current belief that the RNA 3'-cleavage activity of C11 is essential, we show that the NTD-L alone can provide the essential function of *Saccharomyces cerevisiae* C11 whereas classic point mutations in the CTD of full-length C11 (D91A, E92A) that block cleavage, *interfere* with active site function and are toxic to growth. Biochemical and *in vivo* analysis including of the C11 invariant central linker that incorporates information from recent cryo-EM structure results led to a model for Pol III termination-associated reinitiation-recycling. The C11 NTD and CTD stimulate termination and RNA 3'-cleavage respectively whereas the reinitiation-recycling activity unique to Pol III requires only the NTD-linker. The CTD and its RNA 3'-cleavage activity promotes growth rate but is nonessential while the NTD-L is by conventional assay.

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Mishra, S., and Maraia, R.J. (2019). RNA polymerase III subunits C37/53 modulate rU:dA hybrid 3' end dynamics during transcription termination. Nucleic Acids Res 47, 310-27.

**FttA is a CPSF73 homologue that terminates transcription in Archaea**

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Regulated gene expression is largely achieved by controlling the activities of essential, multi-subunit RNA polymerase transcription elongation complexes (TECs). The extreme stability required of TECs to processively transcribe large genomic regions necessitates robust mechanisms to terminate transcription. Efficient transcription termination is particularly critical for gene-dense bacterial and archaeal genomes in which continued transcription would necessarily transcribe immediately adjacent genes and result in conflicts between the transcription and replication apparatuses; the coupling of transcription and translation[7,8] would permit the loading of ribosomes onto aberrant transcripts. Only select sequences or transcription termination factors can disrupt the otherwise extremely stable TEC and we demonstrate that one of the last universally conserved archaeal proteins with unknown biological function is the Factor that terminates transcription in Archaea (FttA). FttA resolves the dichotomy of a prokaryotic gene structure (operons and polarity) and eukaryotic molecular homology (general transcription apparatus) that is observed in Archaea. This missing link between prokaryotic and eukaryotic transcription regulation provides the most parsimonious link to the evolution of the processing activities involved in RNA 3'-end formation in Eukarya.

# Session 10, Chair: Todd Blevins

**Sara Javidnia** - "Population genetic analyses implicate biogenesis of translation machinery in human ageing"

**Cecelia Harold** - "High-content screen reveals lncRNAs as regulators of nucleolar form and function"

**Stephanie L. Cooper** - "In vitro characterization of RNA polymerase I inhibition by BMH-21"

**Wenjun Fan** - "Widespread germline genetic heterogeneity of human ribosomal RNA genes"

**Stephanie Pitts** - "Identification of an E3 ligase regulating the catalytic subunit of RNA polymerase I"

**Neuton Gorjão** - "POLR1D, a common subunit of RNA polymerase I and III, influences its own expression"

**Kristin Watt** - "RNA Polymerase I and III function in neural crest cell and neuronal development"

**Jodie R. Malcolm** - "Widespread Association of ERα with tRNA genes in MCF-7 cells and primary breast tumors"

**Rita Ferreira** - "RNA polymerase I and II inhibitors act synergistically as cancer therapeutic"

**Population genetic analyses implicate biogenesis of translation machinery in human ageing**

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Reduced provision of protein translation machinery promotes healthy ageing in a number of animal models. In humans, however, inborn impairments in translation machinery are a known cause of a range of developmental disorders, collectively called ribosomopathies. Here, we employ population genetic approaches to investigate if adult, tissue-specific biogenesis of translation machinery drives human ageing. We assess naturally occurring variation in the expression of genes encoding subunits specific to the two RNA polymerases (Pols) that transcribe ribosomal and transfer RNAs, namely Pol I and III, and the variation in expression of ribosomal protein (RP) genes, using Mendelian Randomisation. We find each causally associated with human longevity (ß=-0.15±0.047, $p=9.6x10^{-4}$; ß=-0.13±0.040, $p=1.4x10^{-3}$; ß=-0.048±0.016, $p=3.5x10^{-3}$, respectively). These associations do not appear to be mediated by altered susceptibility to a single disease. Interestingly, we find that reduced expression of Pol III, RPs or Pol I promote longevity from different organs, namely visceral adipose, liver and skeletal muscle, echoing the tissue-specificity of ribosomopathies, and we provide evidence that Pol I and RPs may act from organs where their expression is limiting. Our study demonstrates the utility of leveraging genetic variation in expression to elucidate how essential cellular processes impact human ageing. Our findings extend the evolutionary conservation of protein synthesis as a process that drives animal ageing to include humans.

**High-content screen reveals lncRNAs as regulators of nucleolar form and function**

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Ribosome biogenesis, the making of ribosomes in the cell nucleolus is a complex and essential process for all living cells. Our laboratory developed a robust high-throughput assay for nucleolar number that reports nucleolar function (Farley-Barnes et al, 2018) that we have applied to discover long non-coding RNAs (lncRNAs) whose depletion interferes with nucleolar function in human cells. lncRNAs are also increasingly being discovered as viable biomarkers in different human diseases. To date, no systematic screen for novel lncRNA regulators of ribosome biogenesis has been conducted. We performed a high-content screen using a library of 3,940 siRNAs against annotated lncRNAs in human cells, in triplicate. This screen resulted in the discovery of a total of 89 high-confidence hits that alter nucleolar form and function. We used strict cutoffs for mean percent effect compared to controls and strictly-standardized mean difference (SSMD), which is a powerful statistical analysis for effect size. Specifically, we found that 72 siRNAs that knock down lncRNAs caused a significant decrease in nucleolar number and 17 siRNAs caused a significant increase in nucleolar number. We performed network analyses on the total hitlist to identify clusters of proteins that interact with the lncRNAs. One such interaction network is a cluster of homeobox (HOX) lncRNAs, antisense to the HOXA and HOXC genes, transcription factors responsible for axial patterning. A number of clusters are further linked through their interactions with DEADbox (DDX proteins), such as DDX3X and DDX6, which are RNA helicases, the former of which causes a condition known as DDX3X syndrome. Future work will include assays to determine the role these lncRNAs directly play in rDNA transcription, rRNA processing, and global protein synthesis.

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## *In vitro* characterization of RNA polymerase I inhibition by BMH-21

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In highly proliferative human cancer cells, the increase in ribosome biogenesis is predicated on the upregulation of RNA polymerase I (Pol I) transcription of ribosomal DNA (rDNA). Thus Pol I has emerged as a validated target for anti-cancer therapeutic agents. One such agent, the DNA intercalator BMH-21, has been identified to effectively inhibit Pol I transcriptional activity both *in vitro* and *in vivo.* The precise mechanism of inhibition, conserved among eukaryotic species in *Saccharomyces cerevisiae*, mice, and humans, has yet to be fully elucidated. Previous *in vitro* studies with treatment of BMH-21 have demonstrated decreased Pol I elongation rate and processivity, suggesting an inhibitory effect on the elongation phase of transcription. In this study, BMH-21-mediated alterations of nucleotide addition rates have been investigated on Pol I purified from *Saccharomyces cerevisiae*. The effect of BMH-21 treatment on nucleotide addition kinetics characterizes the demonstrated decrease in Pol I elongation rate. Additionally, compromised stability of the elongation complex (EC) has been evaluated as a potential explanation of reduction in Pol I processivity. Treatment with BMH-21 is promising as an initial anti-proliferative agent based upon conserved inhibition of eukaryotic Pol I. Characterization of altered nucleotide addition rates as well as investigation of EC stability further illuminate the mechanisms by which BMH-21 inhibits Pol I activity.

**Widespread germline genetic heterogeneity of human ribosomal RNA genes**

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Polymorphism drives survival under stress and provides adaptability. A vast majority of disease-associated loci represent sequence variations in non-coding regions. rDNA sequences in a single genome are genetically polymorphic due to the internal repeat variation. A considerable amount of sequence heterogeneity has been described even in mature ribosomal (r) RNAs. The impact of rRNA variants affecting physiology and disease is potential broad and extensive, but largely unrecognized and unmeasured. A consensus sequence has been lacking. A reference sequence, U13369, was deposited in 1994 and has been used in vast majority of studies since then. Recent TAR cloning and long-read coding efforts substantially refined the earlier sequence, identified numerous discrepancies and introduced a reference sequence (KY962518) which was 1.8 kb longer than the previous. These sequencing efforts also identified variants (SNV/INDEL) in the coding and IGS sequences based on multiple sequenced clones.

We identified three full and one partial rDNA copies on chromosome 21 (GRCh38) with 99% similarity to the reference KY962518. Pairwise alignment of these copies revealed differences in both length and sequence of the coding sequence. We customized a bioinformatics pipeline for GATK variant calling, and optimized the pipeline for speed and accuracy omitting the need for downsampling. All four rDNA loci on chromosome 21 (total 145 kb) were used for variant calling. We employed whole genome sequencing (WGS) data from the 1000 Genomes Project phase 3 with 2504 individuals from 26 populations. Using the GATK pipeline, we identified a total of 3791 variant alleles. The variants positioned non-randomly on the rRNA gene. Invariant regions included the promoter, early 5' ETS, 5.8S, ITS1 and certain regions of the 28S rRNA, and large areas of the intragenic spacer. 18S rRNA coding region had very few variants. The majority of 28S rRNA variants located on highly flexible human-expanded rRNA helical folds ES7L, ES15L and ES27L, suggesting that these represent positions of diversity and are potentially under continuous evolution. The findings emphasize that the mapping of rRNA genes in the human genomes is at its infancy.

**Identification of an E3 ligase regulating the catalytic subunit of RNA polymerase I**

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RNA polymerase I (Pol I) is upregulated in many cancers, making it a promising target for cancer therapeutics. Yet the mechanisms that govern the stability and regulation of Pol I remain unknown, making it an elusive enzyme to target. Our lab recently discovered a first-in-class small molecule, BMH-21, that blocks Pol I transcription and induces the degradation of the catalytic subunit of Pol I, RPA194. In the process, the Pol I complex disengages from the rDNA. We have observed that treatment with this molecule results in cancer cell-specific cell death. Moreover, the inducible degradation of RPA194 is cancer cell-specific and correlates with cell death. We have found that RPA194 degradation occurs through the ubiquitin proteasome system, and we are studying the mechanisms and regulation of this inducible degradation. A detailed understanding of this process will provide essential knowledge about the stability of the Pol I complex and will benefit therapeutic implementation of Pol I inhibitory strategies.

To identify the E3 ligase involved, we first conducted an RNAi screen for ubiquitin pathway genes involved in RPA194 degradation. This screen identified a Skp-Cullin-F-box protein complex (SCF complex) as a promising candidate E3 ligase. To validate the role of this candidate in RPA194 degradation, we conducted knockout, knockdown, and overexpression analyses. We also examined the interaction of this candidate with RPA194 both in cell lines and *in vitro*. Finally, we analyzed how the expression of this candidate E3 ligase affects the cell response to both BMH-21-mediated RPA194 degradation and cell death. Our results have revealed novel regulatory aspects of Pol I. They have also provided mechanistic insights that support biomarker development and the identification of new therapeutic vulnerabilities.

## POLR1D, a common subunit of RNA polymerase I and III, influences its own expression

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POLR1D is a small subunit that is common to RNA polymerase I and III, which synthesize rRNA and tRNA, respectively. The products of these polymerases are crucial for protein synthesis. *POLR1D* is frequently upregulated in colorectal cancer (CRC) and its high expression is positively correlated with tumour size and poor survival of CRC patients. In contrast, *POLR1D* knock-down inhibits CRC cells proliferation in vitro and tumour-growth in mouse xenograft model. Here we show that that the ectopic overexpression of POLR1D in normal colon cells and several colorectal cancer cell lines decreases the expression of endogenous *POLR1D*. Thus, there is a mechanism that tightly controls the levels of POLR1D protein. However, given high expression of *POLR1D* in CRC cells, the mechanism of *POLR1D* self-regulation is most likely defective in these cells, which may potentially contribute to their tumorigenic properties. The aim of this work therefore is to decipher the mechanism of *POLR1D* self-regulation.

Our data show that downregulation of endogenous POLR1D is not a consequence of either altered levels of its transcript or protein degradation at the proteasome or by autophagy. Instead, polysome profiling experiments show decreased levels of endogenous *POLR1D* mRNA that is associated with ribosomes, suggesting that *POLR1D* may regulate its own expression at the level of translation.

## RNA Polymerase I and III function in neural crest cell and neuronal development

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Ribosome biogenesis is an essential and global process required for cell growth and proliferation. Disruptions in ribosome biogenesis lead to a group of disorders termed ribosomopathies, which display distinct, tissue-specific phenotypes. Perturbations in Pol I and III, which are important for the transcription of rRNAs and non-coding RNAs, may result in craniofacial anomalies, limb anomalies, or neurological defects. To understand the mechanisms underlying the tissue-specificity of these phenotypes, we examined zebrafish with mutations in the Pol I and III subunits *polr1a* and *polr1c*. RNA-Seq analysis of mutant embryos revealed alterations in genes involved in craniofacial and neuronal development. We previously showed that *polr1a* and *polr1c* are necessary for rRNA transcription and neural crest cell survival in craniofacial development, but how these mutations alter nervous system development remains unresolved. At 24 hours post fertilization (hpf), we observed cell death throughout the neural tube in *polr1a* and *polr1c* mutant zebrafish. We hypothesized that this domain of cell death would disrupt formation of multiple neural tube derivatives, including neural crest cells and oligodendrocyte progenitors, leading to craniofacial and myelination defects. Consistent with this, *olig2* expression is diminished prior to 36 hpf and immunostaining for HuC revealed multiple neuronal deficits at 5 dpf in *polr1a* and *polr1c* mutant zebrafish. Distinguishing the roles for *polr1a* and *polr1c* in the presentation of craniofacial versus nervous system phenotypes in the future will further our understanding of the tissue-specificity of Pol I and III dysfunction in human development and disease.

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**Widespread Association of ERα with tRNA genes in MCF-7 cells and primary breast tumours.**

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The oestrogen receptor (ERα) is a potent driver of hormone responsive breast cancer development. Regulating gene expression of target genes such as cyclin D1 and c-Myc, the ERα has a critical role in regulating cell cycle progression and tumour proliferation. While much is known about ERα regulation of RNA polymerase II (Pol II) -transcribed genes, little is understood about how the ERα may prime transformed cells for increased protein biosynthesis, by directly impacting RNA polymerase III (Pol III) transcription of tRNA genes. In 2011, Hah *et al*. identified rapid changes in tRNA gene transcription following β-estradiol stimulation in the MCF-7 cell line [1]. Exciting work from the Shuping Zhong lab revealed that the ERα mediates increased induction of Pol III transcribed genes, $tRNA^{Leu}$ and 5S rRNA, following alcohol treatment, with increased occupancy of the ERα at these promoters [2, 3]. This induction of Pol III transcription is repressed by tamoxifen, further implicating ERα as a Pol III transcription regulator [4]. To understand the possible mechanisms behind ERα-mediated regulation of tRNA gene transcription, we have utilized readily available ERα ChIP-seq datasets from the MCF-7 cell line, in which a high number of tRNA genes were found to be in association with the ERα. This finding was further corroborated when primary breast cancer tumours were similarly studied. Sequence analysis revealed there are no canonical estrogen response elements (EREs) present within 10kbs of these genes, suggesting the mechanism behind ERα recruitment and potential regulation is likely mediated by protein-protein interactions. Our aim is to characterise these putative mechanisms of ERα recruitment and determine how the steroid receptor may contribute to the regulation of tRNA gene transcription.

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**RNA polymerase I and II inhibitors act synergistically as cancer therapeutics**

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CX-5461 is a RNA Polymerase I (Pol I) transcription inhibitor shown to efficiently and specifically target cancer cells both *in vitro* and *in vivo*. Pol I inhibition leads to perturbation of rRNA synthesis that activates the Nucleolar Stress Pathway (NSP) pathway leading to p53 stabilization and consequent cell death, growth arrest and/or senescence. With the aim of identifying therapeutics that can be used in combination with CX-5461 to improve therapeutic efficiency and delay the development of acquired resistance to single agent therapy we screened a library of FDA approved compounds for their ability to exacerbate the effect of Pol I inhibition on the activation of the NSP. Several classes of compounds were identified including Topoisomerase, HSP90, Aurora kinase and CDK inhibitors.

The pan-CDK inhibitors Dinaciclib and Flavopiridol have been shown to inhibit RNA Polymerase (Pol II) activity by preventing the phosphorylation of serine 2 by CDK9 and have reached Phase 2 clinical trials for haematopoietic malignancies. We show that combinatorial therapy with CX-5461 and the CDK9 inhibitors Flavopiridol and Dinaciclib significantly increases the survival of acute myeloid leukaemia (AML) models *in vivo*. This effect is due to an increase in apoptosis. The potential combinatorial therapeutic effect was also observed in *ex vivo* cultures of primary AML patient samples.

Our data indicates that CX-5461 in combination with CDK9 inhibitors can be used in as a therapy to extend survival and reduce acquired resistance. Furthermore, lower doses of CDK9 inhibitors can be used therefore reducing secondary effects.

# Session 11, Chair: Julie Law

**Abu Musa Md Talimur Reza** - "Investigation of the effects of tRNA genes knock-out in human cells"

**Fabian Blombach** - "Cbp1 chromatinisation regulates transcription of CRISPR arrays"

**Breanna R. Wenck** - "Archaeal chromatin dynamics regulate the transcription apparatus"

**Andrew Loffer** - "Features of Pol IV-RDR2 dsRNAs dictate alternative DCL3-dicing patterns in the biogenesis of siRNAs guiding RNA-directed DNA methylation"

**Feng Wang** - "23-nt siRNAs function as passenger strands for Arabidopsis AGO4-associated 24 nt siRNAs and are released by slicing"

**Dany Sibai** - "Transcription Termination Factor 1 (TTF1), a multifunctional regulator of ribosomal RNA gene activity and cell growth"

**Investigation of the effects of tRNA genes knock-out in human cells**

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**Abstract**

In mammalian cells, there are multiple copies of tRNA genes within the same tRNA isotype or isoacceptor families. Curiously, not all of these tRNA genes are equally transcribed, and many of them being completely silent. The full understanding of how individual tRNA genes are regulated is still missing. Among a few hypotheses that were posed is the influence of chromatin context, the existence of proximal Pol II transcriptional unit, and/or the strength of the terminator downstream of a tRNA gene body. Moreover, it is not known whether the expression of the tRNAs is coordinated as a group, at the isoacceptor family level, or, in other words, whether the cells activate low or non-expressed tRNA genes when the active fail to sustain the required cellular needs.

The aim of this work is to test whether: 1) downregulation of active tRNA genes within isoacceptor family leads to activation of the silent ones; 2) genomic manipulation of the terminator sequence affects tRNA gene activity *in vivo*; 3) translocation of silent tRNA into the active chromatin context activates its transcription?

In order to address these questions we employed CRISPR/Cas9-based approach to completely remove individual tRNA genes within $tRNA^{Trp(CCA)}$ family or to manipulate downstream terminator sequences directly in the genome of human cancer cells. We also used retroviral infection to transfer silent tRNA gene into the open chromatin context.

We were able to knock-out four active $tRNA^{Trp(CCA)}$ genes, leaving only one active and two inactive genes. We could not observe any changes in Pol III occupancy at the remaining genes, suggesting no coordinate regulation of these tRNA genes. Strikingly, only one active $tRNA^{Trp(CCA)}$ gene is enough to maintain normal biological functions of human cells.

Furthermore, genetic manipulation of the terminator sequence (removal or introducing) showed no impact on Pol III binding at the tRNA genes. Finally, we did not observe activation of silent $tRNA^{Trp(CCA)}$ after its translocation into open chromatin context. These data suggest the other mechanism(s) control activity of tRNA genes *in vivo*.

**Cbp1 chromatinisation regulates transcription of CRISPR arrays**

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CRISPR arrays are a central part of the CRISPR adaptive immune systems in prokaryotes and serve as templates for the transcription of long non-coding pre-crRNAs that feature regularly spaced repeat sequences. The archaeon *Sulfolobus solfataricus* harbours multiple CRISPR arrays spanning several kilobases and a DNA-binding protein dedicated to the chromatinization of CRISPR arrays termed Cbp1. It remains unknown how Cbp1 affects pre-crRNA transcription from these long CRISPR arrays. We generated a near-nucleotide resolution genome-wide map of Cbp1 occupancy using ChIP-seq and ChIP-exo and studied its effects on transcription elongation by synchronised in vitro transcription assays. We show that Cbp1 recruits the general chromatin protein Cren7 to CRISPR arrays and suppresses spurious transcription from cryptic CRISPR array-internal promoters. In vitro, Cbp1 induces transcriptional pausing upstream of the CRISPR repeat sequences. Our results suggest that Cbp1 plays an important role in the coordinated expression of the *S. solfataricus* CRISPR machinery.

**Archaeal chromatin dynamics regulate the transcription apparatus**

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Most archaeal genomes are organized with histone proteins that have obvious homology to the core eukaryotic histones. Histone-bound chromatin organizes the genome which ultimately regulates the binding and progression of the transcription apparatus. Archaeal histone-based chromatin is often assembled from single histone proteins, although heterodimers of histone isoforms can also generate archaeal histone-based chromatin polymers wherein the DNA is forced into a tightly packed, super-helical structure that retains the same geometry as the DNA within eukaryotic nucleosomes. Substitutions within key regions of archaeal histones are known to (i) disrupt or strengthen histone-DNA binding, (ii) disrupt or strengthen histone-histone interactions, and (iii) disrupt the stability and formation of tightly packed superhelical archaeal histone-based chromatin structures. While disruption of native histone-histone interactions has been examined *in vivo* – wherein modification of archaeal histone-based chromatin polymers results in modified gene expression, disrupted chromatin structure, and disturbed cellular fitness – the impact of individual histone variants and histone isoforms on discrete steps of the archaeal transcription cycle have not yet been resolved. The physicochemical properties of archaeal histone-DNA interactions that direct the formation of three-dimensional chromatin structures and ultimately gene expression are the subject of this investigation. Here, using purified *in vitro* transcription assays, we target individual points of histone-histone/histone-DNA contacts and detail the significance each contact has on regulating the transcription apparatus. Additionally, we introduce techniques to capture and analyze nascent transcripts from archaeal cells with native and variant histone-based chromatin-landscapes to delineate the impacts and regulation imposed by discrete histone-DNA interactions *in vivo*.

**Features of Pol IV-RDR2 dsRNAs dictate alternative DCL3-dicing patterns in the biogenesis of siRNAs guiding RNA-directed DNA methylation**

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## Abstract

In plants, RNA-directed DNA methylation (RdDM) leads to transcriptional silencing of transposons, DNA viruses and a subset of genes. The process begins with transcription of DNA into short (~25-35 bp) double-stranded (ds) RNAs by the coupled activities of DNA-dependent RNA polymerase IV (Pol IV) and RNA-dependent RNA polymerase 2 (RDR2). DICER-LIKE3 (DCL3) then cuts the dsRNAs into short interfering RNA (siRNA) duplexes whose strands can be 23 or 24 nt in length. We show that molecular features of Pol IV and RDR2 transcripts dictate how DCL3 cuts the duplexes, with Pol IV initiating nucleotide bias and RDR2-dependent 3' overhangs, generated in two different ways, influencing which end of the precursor duplex is diced. Resulting alternative dicing patterns explain how 23 or 24 nt siRNAs can be derived from both the Pol IV and RDR2-transcribed strands of siRNA precursor dsRNAs.

**23-nt siRNAs function as passenger strands for *Arabidopsis* AGO4-associated 24 nt siRNAs and are released by slicing**

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In eukaryotes, small RNAs associate with Argonaute proteins to mediate transcriptional or post-transcriptional gene silencing. In plants, the major transcriptional gene silencing pathway is RNA-directed DNA methylation (RdDM), guided by ARGONAUTE4 (AGO4)-associated 24 nucleotide (nt) short-interfering RNAs (siRNAs). These siRNAs are generated by DICER-LIKE3 (DCL3)-cleavage of longer double-stranded RNAs (dsRNAs) whose first strands are synthesized by DNA-dependent NUCLEAR RNA POLYMERASE IV (Pol IV) and whose second strands are synthesized by RNA-DEPENDENT RNA POLYMERASE2 (RDR2). Importantly, DCL3 generates abundant 23 nt siRNAs in addition to 24 nt siRNAs, both *in vivo* and *in vitro*, but the functions of the 23 nt siRNAs are unknown. A hallmark of 23-nt RNAs is the frequent occurrence of an untemplated nucleotide added to the 3' end of RDR2 transcripts by a terminal nucleotidyltransferase activity intrinsic to RDR2. This terminal transferase activity has an important function, modifying the Pol IV-RDR2 transcribed dsRNAs such that DCL3 cleavage yields paired 24 nt and 23 nt RNAs with asymmetric 3' overhangs. We present biochemical and bioinformatic evidence that these asymmetric siRNA-duplexes are loaded into AGO4. The 24 nt siRNA then guides the slicing and release of the 23 nt RNA, which is thus deduced to have functioned as the passenger strand during AGO4 loading. The result is that 24 nt siRNAs whose 5' ends correspond to 5' ends of Pol IV transcripts become stably associated with AGO4 whereas the paired 23 nt RDR2-derived strands are eliminated. We further show that AGO4-24 nt complexes can slice longer complementary RNAs, suggesting that they do so at target loci subjected to RdDM.

**Transcription Termination Factor (TTF1), a multifunctional regulator of ribosomal RNA gene activity and cell growth**

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Transcription Termination Factor I (TTF1) is a Myb-family sequence specific factor whose unique N-terminal negative regulatory domain (NRD) inhibits DNA binding. TTF1 is the ortholog of Reb1p, one of two essential Nucleosome Free Regions (NFRs) inducers in yeast. Mouse and human TTF1 are believed to be responsible for termination of 47S pre-ribosomal RNA (47S rRNA) transcription and for imposing a unidirectional mode of DNA replication across the ribosomal RNA gene (rDNA) repeats. TTF1 has also been implicated in rDNA silencing via a lncRNA, chromatin remodelling and DNA CpG methylation (meCpG) as well as in mediating p14/19-ARF tumour suppressor activity. Moreover, studies have demonstrated a potential role for TTF1 in nucleosome positioning and transcriptional activation. However, to date these potential functions have not been directly tested *in vivo*.

We have now generated mouse cell lines conditionally expressing TTF1. Since loss of TTF1 was expected to cause inviability, an epitope tagged, doxycycline inducible *ttf1* cDNA was first introduced into the NIH3T3 mouse cell line and then CRISPR/Cas9 was used to induce homozygous deletions in the endogenous *ttf1* alleles. The resulting cell lines have been used to determine the role TTF1 plays in cell growth, regulation of lncRNA synthesis and rDNA silencing, and how these functions are inter-related. To do this we have applied a range of techniques including RNA metabolic labeling, psoralen accessibility crosslinking, lncRNA mapping and chromatin immunoprecipitation (ChIP-qPCR and DChIP-Seq). We show that TTF1 protein levels determine the cell proliferation rate by regulating ribosome production and total cellular RNA levels rather than rDNA silencing. The data further show that the lncRNA is indeed generated from the enhancer-associated “Spacer” promoter and suggest an alternative scenario for lncRNA function in which the rDNA activity is regulated by promoter occlusion.

# Session 12, Chair: Olivier Gadal

**Joachim Griesenbeck & Herbert Tschochner** - "The lobe binding subunits of RNA polymerase I cooperate to transcribe efficiently"

**Ann-Kristin Östlund Farrants** - "Chromatin changes in the regulation of RNA pol I transcription"

**Guillermo Abascal-Palacios** - "Structural basis of Ty3 retrotransposon targeting of RNA polymerase III-transcribed genes"

**The lobe binding subunits of RNA polymerase I cooperate to transcribe efficiently nucleosomal and non-nucleosomal templates**

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Eukaryotic nuclear DNA-dependent RNA polymerases (Pols) have evolved to transcribe a chromatin template. However, the major component of chromatin, the nucleosome, may constitute a barrier to transcription. Accordingly, yeast Pol II transcription through reconstituted nucleosomes *in vitro* is strongly impaired, whereas yeast Pol I and Pol III transcribe the same nucleosomal template rather efficiently (1). Interestingly, experimental data indicate that Pol I transcription through nucleosomes is supported by its lobe binding subunits Rpa34.5/Rpa49 and Rpa12.2. Within these subunits distinct subdomains are important for Pol I transcription initiation, elongation and nascent transcript cleavage, as shown in dedicated *in vitro* assays with highly purified Pol I enzymes and naked nucleic acid scaffolds ( (1,2), see also short talk by Katrin Schwank). We would like to understand how these functions contribute to nucleosomal transcription.

The highly defined nature of reconstituted nucleosomal templates makes them a suitable model substrate for *in vitro* transcription assays. However, to gain a detailed mechanistic understanding of eukaryotic nuclear transcription, the process must be eventually reconstituted on a native chromatin template. This is especially true for Pol I transcription, since the Pol I multicopy target genes exists in at least two different chromatin states *in vivo* (3). We have developed a system which enables the enrichment of native Pol I templates in distinct chromatin states (see short talk by Sebastian Kruse). These templates can be characterized in their biophysical properties, protein composition and structure (4,5). We present first data on the use of these templates in highly defined *in vitro* transcription reactions with purified endogenous and recombinant factors. We will discuss how the use of this system may help us to derive a more complete picture of this early level of regulation of eukaryotic gene expression.

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**Chromatin changes in the regulation of RNA pol I transcription.**

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We have recently discovered an antagonistic interplay between the chromatin remodellers B-WICH and NuRD upon glucose depletion and refeeding (1). B-WICH, comprising WSTF, the ATPase SNF2h and Nuclear Myosin, activates transcription by establishing an open chromatin state at the promoter in response to glucose stimulation. This state is counteracted by the silencing complex NuRD, which instead establishes a closed chromatin structure. The molecular mechanism behind this switch is poorly understood. Several non-coding RNA molecules are involved in the recruitment of factors and establishment of chromatin states in mouse cells. TTF-1, which recruits remodelling complexes, is also present at the human promoters, but appears to instead of recruiting chromatin remodellers it is important in stabilising factors, such as UBF, at the promoter. In addition, a number of non-coding RNAs from the human rDNA loci have been identified and two of those species, IGS32as and IGS38, are involved in the establishment of chromatin states at the promoter as well as the human spacer promoter. Taken together, this suggests that human cells employ similar mechanism as mouse cells, but use different factors.

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**Structural basis of Ty3 retrotransposon targeting of RNA Polymerase III-transcribed genes**

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Retrotransposons are endogenous elements that have the ability to mobilise their DNA and integrate at different locations in the host genome. In budding yeast, the Ty3 retrotransposon integrates with an exquisite specificity in a narrow window upstream of RNA Polymerase III-transcribed genes, such as the genes of transfer RNAs, representing a paradigm for specific targeted integration.

Here we present the cryo-EM reconstruction at 4.0 Å-resolution of a reconstituted, active Ty3 strand-transfer complex (Ty3 intasome) during integration into a specific tRNA gene bound to the RNA Polymerase III general transcription factor TFIIIB, which is required for Ty3 specific targeting.

The structure unravels the molecular mechanisms underlying Ty3 integration specificity at RNA Polymerase III-transcribed genes and shed lights into the architecture of a retrotransposon integration machinery during the process of strand transfer at a genomic locus. The Ty3 intasome establishes contacts with a region of the TATA-binding protein (TBP), a subunit of TFIIIB, which is blocked by the ubiquitous transcription regulator negative cofactor 2 (NC2) in RNA Pol II-transcribed genes.

A previously unrecognised chromo domain of the Ty3 integrase mediates non-canonical interactions with TFIIIB and the tRNA gene itself, defining with extreme precision the position of the integration site. Surprisingly, Ty3 retrotransposon tethering to TFIIIB topologically resembles LEDGF/p75 transcription factor targeting by HIV retrovirus, highlighting mechanisms of convergent evolution by unrelated mobile elements and host organisms.

The Ty3 intasome-TFIIIB-tRNA gene complex presented here represents a detailed molecular snapshot of a general transcription factor's co-option by a mobile element, resulting in harmless integration into the host genome.

# Session 13, Chair: Craig Pikaard

**Todd Blevins** - "Evolution of Pol IV domains required for the functional docking of transposable element silencing factors"

**Andrzej Wierzbicki** - "Pervasive non-coding transcription as a genome surveillance mechanism"

**Julie Law** - "The CLASSY family controls tissue-specific DNA methylation patterns in Arabidopsis"

**Jeffrey Smith** - "The budding yeast rDNA locus and chromosome III share a common mechanism of condensin and Sir2 recruitment"

**Evolution of Pol IV domains required for the functional docking of transposable element silencing factors**

Bart Rymen[1], Luisa Felgines[1], Christophe Himber[1], Laura Ferrafiat[1], Marcel Böhrer[1], Lauriane Kuhn[2], Johana Chicher[2], Philippe Hammann[2], Todd Blevins[1,*]

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Genome surveillance in plants and mammals involves the targeted methylation and chromatin-level silencing of transposable elements (TEs). Plant RNA polymerase IV (Pol IV) is critical for directing this DNA methylation to specific chromosomal loci. Pol IV transcribes target loci into noncoding RNA precursors for small interfering RNAs (siRNAs) that guide DNA methylation, silence TEs and regulate certain genes. Pol IV partners with SAWADEE HOMEODOMAIN HOMOLOG1 (SHH1) and four SWI/SNF-like CLASSY proteins to facilitate its recruitment and channels its transcripts to RNA-DEPENDENT RNA POLYMERASE 2 (RDR2) to generate dsRNAs. Pol IV is composed of 12 subunits, like its molecular cousin Pol II, with the largest subunit NRPD1 showing the most divergence in functional domains. How Pol IV evolved to partner with the abovementioned consortium of silencing factors remains unclear. To answer this question, we have combined phylogenetic analyses of Pol IV subunits with mutational scanning, LC-MS/MS analyses and confocal fluorescence microscopy in Arabidopsis, searching for the regulatory domains and protein-protein interaction motifs required for Pol IV function. In this work, we identified evolutionarily conserved motifs unique to NRPD1 that are not directly required for Pol IV's catalytic activity. We will discuss the effect of these non-catalytic motifs on the Pol IV interactome and their role in protecting plant genome integrity.

**Pervasive non-coding transcription as a genome surveillance mechanism**

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Eukaryotic genomes are pervasively transcribed, yet most transcribed sequences lack conservation or known biological functions. In *Arabidopsis thaliana* a specialized RNA polymerase V (Pol V) produces non-coding transcripts, which base-pair with small interfering RNA (siRNA) and allow specific establishment of RNA-directed DNA methylation (RdDM) on transposable elements. We found that Pol V transcribes much more broadly than previously expected, including subsets of both heterochromatic and euchromatic regions. At already established RdDM targets Pol V and siRNA work together to maintain silencing. In contrast, many euchromatic sequences do not give rise to siRNA but are covered by low levels of Pol V transcription, which is needed to establish RdDM *de novo* if a transposon is reactivated. We propose a model where Pol V surveils the genome to make it competent to silence newly activated or integrated transposons. Our model predicts that pervasive transcription of non-conserved sequences may facilitate a broad range of co-transcriptional processes and play an essential role in maintenance of genome integrity.

**The CLASSY family controls tissue-specific DNA methylation patterns in Arabidopsis**

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# current affiliations for M.Z., C.B., and A.L.

DNA methylation shapes the epigenetic landscape of the genome, plays critical roles in regulating gene expression, and ensures transposon silencing. As evidenced by the numerous defects associated with aberrant DNA methylation landscapes, establishing proper tissue-specific methylation patterns is critical. Yet, how such differences arise remains a largely open question in both plants and animals. Here we demonstrate that CLASSY1-4 (CLSY1-4), four locus-specific regulators of DNA methylation that are differentially expressed during plant development, play major roles in controlling tissue-specific DNA methylation patterns. Depending on the tissue, the genetic requirements for specific CLSYs differ significantly and, on a global scale, certain *clsy* mutants are sufficient to largely shift the epigenetic landscape between tissues. Together, these findings not only reveal substantial epigenetic diversity between tissues, but assign these changes to specific CLSY proteins, revealing how locus-specific targeting combined with tissue-specific expression enables the CLSYs to generate epigenetic diversity during plant development.

Zhou M, Coruh C, Xu G, Bourbousse C, Lambolez A, Law J. The CLASSY family controls tissue-specific DNA methylation patterns in Arabidopsis. 2021 January 01; :2021.01.23.427869. http://biorxiv.org/content/early/2021/01/24/2021.01.23.427869.abstract
DOI:10.1101/2021.01.23.427869

**The budding yeast rDNA locus and chromosome III share a common mechanism of condensin and Sir2 recruitment**

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Condensin plays an important and evolutionarily conserved role in mitotic chromosome condensation, three-dimensional genome organization and regulation of gene expression. Condensin is a multi-subunit complex that has a natural affinity for the promoters of highly transcribed genes, and also associates with specific transcription factors. However, a general mechanism for functional loading of the complex onto chromatin remains elusive. Our lab previously demonstrated that in haploid *MATa* yeast cells, condensin and Sir2 (a histone deacetylase) both associate with the recombination enhancer (RE), a cis-acting element on chromosome III that directs donor preference of mating-type switching. Here, Sir2 locally regulates transcription of a small gene called *RDT1*, while condensin contributes to 3-dimensional organization of chromosome III and mating-type switching, indicating the *RDT1* promoter acts as a locus control region (LCR). We have now characterized the mechanism of Sir2 and condensin recruitment to the LCR, uncovering a critical role for a non-meiotic version of the monopolin complex, known as cohibin (Lrs4 and Csm1 subunits), and analogous to its known role in recruiting condensin to the rDNA locus. To test if cohibin functions more generally in condensin loading, or as a condensin accessory factor, we performed ChIP-seq for the Brn1 subunit in WT and *lrs4Δ* strains. Numerous Brn1 peaks (including the *RDT1* promoter LCR and rDNA) were eliminated or significantly reduced in the absence of Lrs4, consistent with a condensin loader hypothesis. Micro-C XL was then used to characterize the global effects of defective condensin recruitment on chromosome conformation in a *lrs4Δ* mutant, or when Brn1 was rapidly depleted using an auxin-inducible degron. Significant alterations were observed on multiple chromosomes, though the most severe changes occurred on chromosome III, consistent with observed defects in mating-type switching efficiency and donor preference. We further found that deleting the nucleolar DNA replication fork blocking protein Fob1 unexpectedly prevented association of Lrs4 and condensin with the *RDT1* promoter, pointing to a potentially broader role for Fob1 in chromosome structure-function beyond the rDNA locus which is also evident from Micro-C XL in a *fob1Δ* mutant. We hypothesize that cohibin (Lrs4/Csm1) is a condensin loader targeted to the *RDT1* promoter region by Fob1, where condensin establishes specialized chromosome III architecture through a loop extrusion mechanism.

# Session 14, Chair: Christoph Engel

**Alicja Armatowska** - "The connection between Maf1, a negative regulator of RNA polymerase III, and translation in yeast"

**Hitha Gopalan Nair** - "RNA polymerase III inhibition affects cytotoxic and tumour promoting effects of TNFα"

**Aneta Jurkiewicz** - "MAF1 is involved in the regulation of RNA polymerase III activity in macrophages upon LPS treatment"

**Christopher Schächner** - "A system to study yeast RNA Polymerase I pre-initiation complex assembly in vivo – A “mini” story of cis-elements and transcription factors"

**Tamara Potapova** - "Anticancer compound library screen identifies Cdk inhibitors as novel inducers of nucleolar stress"

**Adriana Coke** - "Transcription by the Chloroplast-encoded RNA Polymerase May Determine Structural Organization of the Chloroplast Nucleoid"

**Hazel Mangan** - "NEAT; The combined power of NOR editing and chromosome transfer to study RNA polymerase I transcription, rRNA processing and human nucleoli"

**Anastasia McKinlay** - "Determination of ribosomal RNA gene repeat organization within Arabidopsis Nucleolus Organizer Regions by long-range sequencing and dotplot puzzle fitting"

**The connection between Maf1, a negative regulator of RNA polymerase III, and translation in yeast**

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Maf1, as a master regulator of RNA polymerase III (Pol III) transcription, represses the synthesis of tRNAs under conditions that are detrimental to yeast growth. Our previous study showed that the export machinery is saturated in the yeast *maf1*Δ mutant due to the increased amount of tRNA precursors (1). Furthermore, *maf1*Δ cells have insufficient levels of modification enzymes to modify the increased amount of tRNAs (2). Presumably, the unbalance in the levels of mature functional tRNAs exerts an effect on the growth and translation in *maf1*Δ cells. Besides the *maf1*Δ growth defect in the medium containing a nonfermentable carbon source, our studies showed an additional phenotype in the fermentable medium. *maf1*Δ cells needed more time than the wild type to start re-proliferation into fresh liquid glucose medium while in the exponential phase, the doubling time of the wild type and mutant strain were comparable. *in vivo* labeling of translation products in the *maf1*Δ mutant revealed decreased levels of cytosolic translation when compared to the control wild type strain. Additionally, the link between Maf1 and translation was identified by a genetic approach in which the *TEF1* and *TEF2* genes were cloned as suppressors of the *maf1*Δ growth defect in a medium containing a nonfermentable carbon source. Both *TEF* genes encode a translation elongation factor, eEF-1α, responsible for the delivery of aminoacyl-tRNAs to the ribosome. eEF-1α is also implicated in tRNA nuclear export. Our studies showed that cytosolic translation levels in *maf1*Δ cells were increased by *TEF* overdose. In addition, overexpression of *TEF* resulted in a shorter delay in restarting proliferation. We suggest that formation of the tRNA/eEF-1α complex during translation elongation is disturbed in the *maf1*Δ mutant. Possibly, the overexpression of *TEF1/2* genes restores the balance between tRNA and eEF-1α. Alternatively, *TEF* overdose causes an increase in tRNA export. Knowing that the *maf1*Δ cells accumulate pre-tRNAs in the nucleus, the increase of nuclear export by the overproduced eEF-1α would be advantageous to enhance the cytoplasmic tRNA pool involved in translation.

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**RNA polymerase III inhibition affects cytotoxic and tumour promoting effects of TNFα**

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Tumour necrosis factor alpha (TNFα) is a multifunctional cytokine playing a key role in apoptosis, cell survival as well as in inflammation and immunity. Although named for its antitumor properties, TNFα has also tumour promoting properties. TNFα is often present in large quantities in tumours, and cancer cells frequently acquire resistance to this cytokine. In consequence, TNFα may increase the proliferation and metastatic potential of cancer cells. TNFα-driven increase in metastasis is a result of the ability of this cytokine to induce epithelial-to-mesenchymal transition (EMT).

Elevated RNA polymerase III (Pol III) activity is a recurring feature of many tumours. The literature data show, however, that high Pol III activity alone is not sufficient to promote tumorigeneses. On the other hand, inhibition of Pol III inhibits cancer cell proliferation and migration. Thus, leaving an open possibility to use Pol III as a target for therapeutic intervention.

The data from our laboratory show that inhibition of Pol III affects inflammatory signalling in immune cells. Whether it is also valid for non-immune cells is not known. The aim of this study, therefore, is to investigate whether inhibition of RNA polymerase III activity can influence the impact of TNFα on colorectal cancer cells.

Here we show that in TNFα-sensitive colorectal cancer cells, Pol III inhibition augments the cytotoxic effect of this cytokine. On the other hand, in TNFα-insensitive cells, inhibition of Pol III blocks TNFα-induced proliferation, migration and expression of EMT related proteins.

Many of the phenotypes induced by TNFα are mediated by NF-κB transcription factor. NF-κB is a key transcription factor mediating inflammatory signals and has been also suggested to have a wide-ranging role in tumour progression, including acceleration of cell proliferation, inhibition of apoptosis, promotion of cell migration and metastasis. Our data show that Pol III inhibition blocks NF-κB activation upon TNFα treatment, thus potentially suggesting the mechanism of Pol III inhibition-driven sensitization of cancer cells to this cytokine.

**MAF1 is involved in the regulation of RNA polymerase III activity in macrophages upon LPS treatment**

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Macrophages, cells of the innate immune system, have many biological functions, such as antigen presentation, target cell cytotoxicity, phagocytosis, and regulation of inflammation. Activation of macrophages with lipopolysaccharides (LPS), a major component of the outer membrane of most Gram-negative bacteria, induces rapid transcriptional changes and, within a few hours, transcription of several hundred genes is altered, these include cytokines and other inflammation mediators. This transcriptional activity is followed by an extensive increase in translation, which in turn requires an increased level of tRNAs, that are transcribed by RNA polymerase III (Pol III).

Here we show that in macrophages stimulated with LPS, Pol III activity is regulated by its negative regulator, MAF1, and upon LPS treatment, MAF1 is rapidly phosphorylated in an mTOR-dependent manner. We also show that mTOR activity is required to activate Pol III in macrophages stimulated with LPS. The results obtained by us imply that the mTOR-MAF1 pathway is a major mechanism of Pol III regulation in macrophages stimulated with LPS.

## A system to study yeast RNA Polymerase I pre-initiation complex assembly *in vivo* – A "mini" story of cis-elements and transcription factors

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The most abundant RNA in eukaryotes are the ribosomal (r)RNAs. In the model eukaryote *S. cerevisiae* (hereafter called yeast) the large 35S rRNA precursor molecule coding for three out of four of the mature rRNAs is transcribed by RNA polymerase (Pol) I from a multi-copy gene locus. The 35S rRNA gene is the only target of Pol I which recognizes the inherent promoter by a dedicated set of transcription factors. Pol I and the rDNA have been a model locus since the beginning of molecular biology, but the detailed mechanism and molecular basis of how Pol I achieves this high transcriptional output are still not fully understood. Current knowledge has been gained from analyzing Pol I transcription using more or less defined reconstituted systems *in vitro*, but also genetic, molecular and cell biological approaches *in vivo*. The latter often suffered from the fact, that Pol I transcription is an essential process, which – when perturbed – may result in drastic indirect effects. Here we present an in vivo approach using a yeast strain which does not depend on Pol I and its specific transcription machinery since it synthesizes 35S rRNA by Pol II from a multicopy plasmid. Furthermore, in this strain the chromosomal multicopy rRNA gene locus has been deleted. Using this genetic background Pol I pre-initiation complex (PIC) assembly can be studied on a minigene containing all known functional genetic elements responsible for efficient Pol I transcription.To investigate Pol I transcription at the minigene we employ Chromatin Endogenous Cleavage (ChEC) to analyze chromatin composition, psoralen photo-crosslinking to study chromatin structure, and RTqPCR to measure RNA levels. We find that the minigene recapitulates all hallmarks of chromosomal 35S rRNA gene transcription. We use the system to further explore the role of 35S rRNA gene promoter cis-regulatory elements as well as the consequences of specific mutations in components regarding Pol I PIC assembly. Our results validate this system as source for templates to study RNA Pol I transcription in a native chromatin context *in vitro* (see short talk by Sebastian Kruse).

**Anticancer compound library screen identifies Cdk inhibitors as novel inducers of nucleolar stress.**

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The nucleolus is a membrane-less organelle assembled around ribosomal DNA transcribed by PolI. Inhibition of PolI transcription induces a canonical nucleolar stress response. In this study, we screened a library of anticancer drugs for compounds that induce morphological changes in nucleolar structure consistent with nucleolar stress. We identified various classes of inhibitors causing nucleolar phenotypes similar to canonical stress response, as well as distinct morphological phenotypes. Several inhibitors of cyclin-dependent kinases (Cdks) induced nucleolar impairment but with very different morphology than the canonical nucleolar stress. Treatment with CDK inhibitors caused rapid and complete dispersal of the granular component of the nucleolus, while the fibrillar center of the nucleolus de-compacted. Under these conditions, POLR1A, the catalytic subunit of the RNA polymerase I complex, disassociated from rDNA almost completely. The rDNA transcription was also abrogated. Washing out the Cdk inhibitors caused a reversal of this phenotype, indicating that the nucleolus can be re-assembled if the kinase activity is restored. We propose that Cdk-dependent phosphorylation may be required for the assembly of functional PolI transcriptional complex on rDNA, and for other protein and RNA interactions within the granular component of the nucleolus.

**Transcription by the Chloroplast-encoded RNA Polymerase May Determine Structural Organization of the Chloroplast Nucleoid**

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Long after its endosymbiosis into eukaryotic plant cells, the chloroplast has retained an independent genome. Many genes required for photosynthesis are still encoded and transcribed within the chloroplast itself. Most of this essential transcription is completed by the chloroplast-encoded RNA polymerase, PEP. Our recent findings suggest that transcription by PEP is tightly correlated with significant protein binding and association with the chloroplast inner membrane. This seems to be in stark contrast to the phenomena of heterochromatin in the nucleus, where tightly-packed and protein-bound regions of DNA inhibit transcription. Even more interesting, our preliminary data suggests that transcription by PEP may control the organization/compaction of chloroplast DNA, instead of the other way around. This is also in contrast to the nuclear model of chromatin packing. How transcription by PEP may affect DNA compaction, and why this is biologically relevant, are still open questions. To answer these questions, it is necessary to determine which proteins may be involved in the spatial organization of chloroplast DNA, and how these nucleoid-associated proteins may be controlled by, or control, transcription. As a result, we are performing mass spectrometry following crosslinking on isolated chloroplast nucleoids, which should reveal a network of protein-protein interactions in the nucleoid. Additionally, we are developing several transgenic lines of potential chloroplast nucleoid-associated proteins with affinity or fluorescent tags, which should allow us to investigate their localization on DNA and independently probe for protein-protein and protein-DNA interactions. Altogether, these experiments aim to produce a clearer picture of the relationship between transcription and DNA organization in the unique model system of the chloroplast.

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**NEAT; The combined power of NOR editing and chromosome transfer to study RNA polymerase I transcription, rRNA processing and human nucleoli.**

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Nucleolar organiser regions (NORs) comprising ribosomal gene (rDNA) arrays are located on the p-arms of the five human acrocentric chromosomes, HSA13, 14, 15, 21 and 22. The use of powerful new genome editing technologies to study human NORs has been hampered by their shared sequence composition and essential role in ribosome biogenesis. To overcome these challenges, we have developed a 2-step workflow which we term NEAT (NOR editing and Transfer) (Mangan and McStay 2021). Remarkably efficient 'scarless' NOR editing is performed using CRISPR/Cas9 across rDNA arrays that are held within mouse mono-chromosomal somatic cell hybrids. In this context, NORs are 'poised', transcriptionally silent due to incompatibility between the mouse SL1 complex and the human rDNA promoter sequences. Yet, the NOR remains bound by murine upstream binding factor (UBF) and associated with the mouse nucleoli. Indeed, transcription of human rDNA arrays (even after extensive genome editing) by murine RNA polymerase I can be reconstituted by ectopic expression of human SL1 components TAFIs A-D in a poly-cistronic inducible system. Next, edited NORs are shuttled into normal human hTERT-RPE1 cells by microcell-mediated chromosome transfer. RNA polymerase I transcription on edited NORs resumes and derived rRNAs can be visualised through all stages of ribosome biogenesis, and into fully functional ribosomes. Thus NEAT provides a platform for reverse genetic approaches to studying all stages of ribosome biogenesis.

Mangan H, McStay B. 2021. Human nucleoli comprise multiple constrained territories, tethered to individual chromosomes. *Genes Dev*. **35:** 449-469

**Determination of ribosomal RNA gene repeat organization within Arabidopsis Nucleolus Organizer Regions by long-range sequencing and dotplot puzzle fitting**

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Many sequenced genomes remain in draft stages due to gaps that are difficult to close. However, closing the gaps is important for studies of gene and chromosome functions in the missing regions. Among the most difficult gaps to close are nucleolus organizer regions, or NORs, which are chromosomal loci at which ribosomal RNA genes transcribed by RNA Polymerase I are repeated in long tandem arrays. Two NORs are present in the *Arabidopsis thaliana* genome, located at the distal ends of Chr2 and Chr4 and thus named *NOR2* and *NOR4*, respectively. In the ecotype Col-0, each NOR is estimated to span ~4 million basepairs and consist of ~375-400 rRNA genes that are each ~10 kb in length. These rRNA genes are nearly identical in sequence, with only limited sequence variation to discriminate one from the other. However, by applying a similarity matrix algorithm to ultra-long Oxford Nanopore reads we were able to visualize patterns of subtle sequence variation within rRNA genes arrays that allow them to be merged with overlapping reads to generate larger contigs, a process we call dotplot puzzle fitting. This approach has allowed us to generate assemblies for the centromere-proximal and telomere-proximal ends of both NORs that are several hundred thousand basepairs in length as well as long contigs internal to the NORs, some exceeding one million basepairs in length. These assemblies allow insights into NOR organization, including the patterns of rRNA gene subtype distribution and the presence of degenerate or transposon-inserted rRNA genes at each end of the NOR that undergoes developmentally regulated silencing, *NOR2*.

# Session 15, Chair: Kate Hannan

**Jessica Finlay-Schultz** - "A novel progesterone receptor-RNA polymerase III association represses estrogen-dependent growth in breast tumor patient-derived xenografts"

**Ian Willis** - "System-wide changes in the metabolism of Maf1 KO mice"

**Kevin Van Bortle** - "A cancer-associated RNA polymerase III identity drives expression of SNAR-A noncoding RNA"

**Trine Mogensen** - "Roles of POL III in antiviral defenses to varicella zoster virus and SARS-CoV2"

**Alan C. Kessler** - "A dual function Pol III-dependent tRNA-gene and activation pathways of the cellular innate immune system"

**A novel progesterone receptor-RNA polymerase III association represses estrogen-dependent growth in breast tumor patient-derived xenografts**

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Progesterone receptors (PR) suppress estrogen receptor (ER)-mediated transcription in breast cancers. However, a mechanistic basis for this repression has been lacking. Recent reports indicate this occurs in part through global repositioning of ER on chromatin in the presence of selective PR modulators (SPRMs), both agonists and antagonists. Using ER+PR+ breast cancer patient-derived xenografts (PDX) in the presence of estradiol (E2) alone or E2 plus the natural hormone progesterone (P4) or a synthetic SPRM medroxyprogesterone acetate (MPA), we demonstrate that SPRMs suppress tumor growth similar to the antiestrogen tamoxifen (a common early-stage breast cancer treatment). In these tumors, P4 and MPA alter up to half of ER regulated genes at the transcript level. However, the majority of these genes (>80%) either show no change in ER chromatin binding by ChIP-seq or have no ER binding sites near their promoter. We made the interesting discovery via PR ChIP-seq that PR (but not ER) localizes at a large fraction of RNA Pol III-regulated tRNA genes. RIME for PR and subsequent IP found that PR associates with the Pol III complex. Furthermore, select pre-tRNA transcripts and mature tRNA pools are decreased in SPRM-treated tumors. These data implicate multifaceted SPRM-mediated control of E2-dependent tumor growth. In addition to antagonizing E regulated genes at the transcription level, SPRMs can regulate translation though depletion of amino acid charged tRNAs, thus slowing protein synthesis. Ongoing studies in PDX and novel PDX-derived cell lines include determining whether PR associates with tRNA genes through direct DNA binding or indirect protein-protein interactions, the role of Brf1 and Maf1 recruitment by activated PR, and how alteration of the tRNA pool may selectively change translational preference through non-optimal codon usage. Identifying which breast tumors utilize this growth suppressive mechanism may pinpoint appropriate candidates for SPRM therapy in advanced tumors and provide a tool for predicting tumor progression.

**System-wide changes in the metabolism of *Maf1* KO mice**

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MAF1 is a master negative regulator of transcription by RNA polymerase (Pol) III. In mammalian systems, MAF1 functions chronically to limit Pol III transcriptional activity in both dividing cell populations and postmitotic tissues[1]. At the same time, MAF1 function can be tuned in response to signaling pathway inputs to further restrict Pol III activity depending on nutrient conditions and exposure to diverse cellular stressors[2]. Whole-body deletion of *Maf1* in mice releases the excess transcriptional capacity of Pol III, increasing transcription from three to ten-fold in all tested tissues in both fed and fasted states. Despite this, the levels of mature tRNA, 5S rRNA and other abundant Pol III transcripts are largely unchanged. These data suggest a futile RNA cycle in which increased Pol III-directed RNA synthesis is offset by increased RNA turnover. Futile RNA cycling is energetically wasteful and is thought to underlie the metabolic inefficiency and lean body composition of *Maf1* KO mice, which also exhibit increased healthspan. Consistent with these changes, lipolysis is increased in white adipose tissue and autophagy/lipophagy is increased in the liver. Targeted metabolite profiling in liver indicates substantial metabolic reprograming (e.g. increased TCA cycling, fatty acid oxidation, urea cycle activity, nucleotide synthesis and nucleotide breakdown)[3]. We reasoned that if futile RNA cycling contributes significantly as a mechanism of whole-body energy expenditure in *Maf1* KO mice, then metabolism should be affected in most, if not all, nucleated cells and tissues. To address this question, we surveyed six tissues for changes in amino acids, biologic amines, acyl carnitines and various lipid species. We also performed widely-targeted profiling of ~600 small hydrophilic metabolites in plasma from fed and fasted mice and in urine. Significant changes in metabolite profiles were seen in all tissues and biofluids and a subset of these shared a metabolic signature - reduced levels of long chain acyl carnitines, consistent with increased fatty acid oxidation. In plasma, 56 unique metabolites were altered suggesting effects on inter-organ metabolic communication and/or clearance of metabolites. Notably, >60 metabolites were differentially present in urine. The levels of amino acids, nucleosides, bases and their modified derivatives (e.g. N6 isopentenyl adenine) were increased in *Maf1* KO urine. The loss of these molecules from the system adds to the energetic cost of life without *Maf1,* helping to account for their lean phenotype, and provides further evidence of elevated tRNA turnover in the mice.

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**A cancer-associated RNA polymerase III identity drives expression of SNAR-A noncoding RNA**

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Dysregulation of the RNA polymerase III (Pol III) transcription program, which synthesizes tRNA and other classes of small noncoding RNA critical for cell growth and proliferation, is associated with cancer and human disease. Previous studies have identified two distinct Pol III isoforms defined by the incorporation of either subunit POLR3G (RPC7$\alpha$) during early development, or POLR3GL (RPC7$\beta$) in specialized tissues. Though POLR3G is re-established in cancer and immortalized cell lines, the contributions of these isoforms to transcription potential and transcription dysregulation in cancer remain poorly defined. Using an integrated Pol III genomic profiling approach in combination with *in vitro* differentiation and subunit disruption experiments, we discover that loss of subunit POLR3G is accompanied by a restricted repertoire of genes transcribed by Pol III. In particular, we observe that a specific class of small noncoding RNA, *SNAR-A*, is exquisitely sensitive to the availability of subunit POLR3G in proliferating cells. Taken further, large-scale analysis of Pol III subunit expression and downstream chromatin features identifies concomitant loss of POLR3G and *SNAR-A* activity across a multitude of differentiated primary immune cell lineages, and conversely, coordinate re-establishment of POLR3G expression and *SNAR-A* features in a variety of human cancers. These results altogether argue against strict redundancy models for subunits POLR3G and POLR3GL, and instead support a model in which Pol III identity itself functions as an important transcriptional regulatory mechanism. Upregulation of POLR3G, which is driven by MYC, identifies a subgroup of patients with unfavorable survival outcomes in specific cancers, further implicating the POLR3G-enhanced transcription repertoire as a potential disease factor.

Van Bortle, K, Marciano, DP, Liu, Q, Lipchik, AM, Gollapudi, S, Geller, BS, Monte, E, Kamakaka, RT, and Snyder, MP. A cancer-associated RNA polymerase III identity drives robust transcription and expression of SNAR-A noncoding RNA. bioRxiv preprint: doi: https://doi.org/10.1101/2021.04.21.440535

## Roles of POL III in antiviral defenses to varicella zoster virus and SARS-CoV2

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In most individuals, varicella zoster virus (VZV) causes varicella upon primary infection and zoster during reactivation. However, in a subset of individuals, VZV may cause severe disease, including infection in the central nervous system (CNS). Recent studies by our group have demonstrated that defects in the immunological DNA sensor RNA polymerase III (POL III) confers impaired antiviral interferon responses and selective increased susceptibility to VZV infection, thus providing fundamental new insight into VZV immunity. Here I will present our data on the identification of functionally defective genetic variants in different subunits of POL III in children as well as in adults causing disease manifestations, including encephalitis, vasculitis, and stoke. The roles of POL III in cellular housekeeping and immune surveillance during VZV infection are described and the latest knowledge on POL III and DNA sensing in VZV infection are discussed. In this context, emerging outstanding questions related to POL III in immunity to VZV and other alphaherpes viruses, and how this new insight may be translated into clinical medicine will be highlighted. Finally, novel results of a possible role of POL III in SARS-CoV-2 immune responses will be presented.

**A dual function Pol III-dependent tRNA-gene and activation of the cellular innate immune system**

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Specific heterozygous mutations to RNA polymerase III (pol III) have recently been shown to cause a specific viral immunodeficiency syndrome to Varicella zoster virus (VZV), leading to severe outcomes of “chicken pox” in children, and in adults with reactivated infection. This has been attributed to the failure of cytoplasmic-Pol III (cy-pol III) to carry out a recently attributed innate immune-specific activity. Certain RNA viruses are detected by cytoplasmic RIG-I, which recognizes 5’-triphosphate RNA and initiates a signaling cascade inducing type I interferon stimulated genes (ISGs). Detection of some DNA viruses, such as VZV, also depends on RIG-I, however, the DNA first undergoes transcription by cy-Pol III, generating the immunostimulatory 5’-triphosphate-RNA. Nuclear-Pol III transcribes tRNA genes and its other essential ncRNA gene substrates as important components of immune cell function. It has also been reported to transcribe immune-signaling RNAs, from a 5S “pseudogene,” tRNA-derived fragments (tRFs), and nc886 RNA. Upon viral infection, these ncRNAs can stimulate the immune system via the ISG response. In summary, nuc-pol III and cy-pol III contribute to innate immune mechanisms.
We discovered a novel bifunctional tRNA gene that when over-expressed in HEK293 cells, produces both a conventional tRNA that promotes mRNA translation and also forms an alternate transcript that activates RIG-I and induces a full set of >200 ISGs. Disruption of RIG-I by siRNA knockdown abolished the ISG response, indicating its role as the primary sensor in detecting this novel RNA. This tRNA gene is unique among its six anticodon gene family members in activity to induce ISGs. Numerous point mutations including to the classic internal promoter elements, terminator, alternative transcription start sites (TSSs), anticodon and other regions have been done together with structure prediction algorithms to decipher a secondary structure of the alternative transcript that induces ISGs. Maximal ISG induction is dependent on use of the first terminator which at 4Ts allows substantial read-through. Remarkably, mutation to 3T ablates ISG induction whereas mutation to 5T or 6T which greatly improves termination efficiency, substantially diminishes ISG induction. Consistent with this, overexpression of the pre-tRNA chaperone La protein decreases ISG induction and siRNA-mediated knockdown of La increases ISG expression. Our data suggest that the TSS used for the alternate RNA structure that induces ISG and the TSS that best forms conventional tRNA differ and the possibility that they may be regulated. The data suggest this tRNA gene has two potential activities, the intra-nuclear tRNA maturation pathway can have an alternate branch that is productive toward ISG induction in the cytoplasm. A survey of ~20 additional tRNA genes of a variety of identities indicate that this gene is one of a small subset that induce ISGs. Thus we have identified a novel class of bifunctional tRNA genes with potential to activate an ISG response whose characterization is in progress.

# Session 16, Chair: Craig Cameron

**Shuping Zhong** - "The role and mechanism of pAMPKα-mediated dysregulation of Brf1 and RNA Pol III genes"

**Ashley Knox** - "RNA polymerase III transcribed gammaherpesvirus non-coding RNAs interact with host proteins and drive pathogenesis"

**Emilio Merheb** - "Defective myelination in an RNA polymerase III mutant leukodystrophic mouse"

**Stefanie Perrier** - "POLR3-related leukodystrophy: Defining novel phenotypes from very mild to extremely severe"

**The role and mechanism of pAMPKα-mediated dysregulation of Brf1 and RNA Pol III genes**

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TFIIB-related factor 1 (Brf1) is a key transcription factor. It specifically regulates transcription of tRNAs and 5S rRNA, which are required for cell transformation and tumor development. Significance and molecular mechanism of upregulation of Brf1, tRNAs, 5S rRNA in lung cancer are unclear. We determined the alteration of Brf1 expression and its significance in human cases of lung cancer. We investigated the relationship of the abnormal expression of Brf1 with high level of phosphorylated AMPKα (pAMPKα) and explored the molecular mechanism of pAMPKα-mediated dysregulation of Brf1 and tRNAs in lung cancer cells. Our studies indicate that Brf1 is significantly overexpressed in the cases of lung cancer. The cases with high Brf1 expression displays short overall survival times. Interestingly, elevation of Brf1 expression of the cases is accompanied with high level of pAMPKα. Brf1 and pAMPKα colocalize in nuclei of lung cancer cells. Further analysis indicates that carcinogen, MNNG induces pAMPKα to upregulate Brf1 expression, resulting in enhancement of tRNAs and 5S rRNA transcription. In contrast, inhibiting pAMPKα activity decreases cellular levels of Brf1, resulting in decrease in Pol III gene transcription to reduce the rates of cell proliferation and colony formation of lung cancer cells. These outcomes demonstrate that high levels of Brf1 expression reveal worse prognosis in lung cancer patients. pAMPKα-mediated dysregulation of Brf1 and tRNAs plays important roles in cell proliferation, colony formation and tumor development of lung cancer. Brf1 is as a biomarker of diagnosis and prognosis of lung cancer. It is a new mechanism that pAMPKα mediates dysregulation of Brf1 and Pol genes to promote lung cancer development.

**RNA polymerase III transcribed gammaherpesvirus non-coding RNAs interact with host proteins and drive pathogenesis**

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Gammaherpesviruses (γHVs) have evolved with their hosts for millions of years and, as such, have become experts in modulating host cells to mediate lifelong infection. One method of achieving this is through the use of non-coding (nc) RNAs. The γHV ncRNAs, including the mouse gammaherpesvirus 68 TMERs and Epstein-Barr virus EBERs, are abundantly expressed and required for pathogenesis. These ncRNAs are transcribed by RNA polymerase III (pol III), lending conserved features to the transcripts such as an uncapped 5' triphosphate end and a 3' poly-U termination sequence, which may be recognized by host innate immune sensors. The TMER locus contains eight TMERs, each with tRNA-like structures followed by stem loops that are processed into miRNAs. Previous work in our lab showed that knock-out of all TMER genes abrogated pathogenesis; however, a single TMER (TMER1) restored pathogenesis, even without the associated miRNAs. These data suggest a sequence-independent function of the TMERs. Considering the similarity in structure across various TMERs, we hypothesized that this function was not unique to TMER1 and that other individual TMERs could restore pathogenesis. To test this, we generated multiple single-ncRNA virus recombinants that express either TMER4, TMER5, TMER8, or EBV EBERs in place of the TMERs. We found that expression of any single TMER, or the EBERs, is sufficient to restore pathogenesis to wild-type virus levels, suggesting sequence-independent functional redundancy across these pol III-transcribed virus ncRNAs *in vivo*. Additionally, we showed that the TMERs and EBERs share the ability to bind to the RNA binding proteins, RIG-I and La/SSB. These interactions may depend on common features of short pol III-transcribed RNAs such as a 5'-triphosphate, regions of dsRNA, and/or the 3'-poly-U termination signal. Current work is focused on testing the extent to which the interaction of these virus ncRNAs with host RNA binding proteins influences acute responses and pathogenesis. These studies reveal the conserved, sequence-independent role of γHV ncRNAs in their interactions with the host.

**Defective myelination in an RNA polymerase III mutant leukodystrophic mouse**

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Leukodystrophies constitute a group of genetically heterogeneous inherited diseases that affect myelin sheath production and/or function in the central nervous system (CNS). RNA polymerase (Pol) III-related leukodystrophy, an autosomal recessive disorder, is the second most prevalent leukodystrophy and was identified when five initially distinct syndromes with overlapping clinical and radiological features were found to have a common molecular basis. Patients are diagnosed between birth and adolescence with neurological and non-neurological deficits and are classified according to distinct brain MRI features and confirmed by sequencing. RNA polymerase (Pol) Pol III-related leukodystrophies involve mutations primarily in *Polr3a* and *Polr3b*, which encode the two largest subunits and form the catalytic core of the enzyme. Pol III synthesizes abundant short non-coding RNAs that have essential functions in protein synthesis, secretion and other processes. Despite the ubiquitous functions of these RNAs, the basis of this neural sensitivity and the mechanisms of disease pathogenesis remain unknown. We show that conditional knock-in of pathogenic *Polr3a* mutations in the Olig2 lineage in mice results in growth, neurobehavioral and hypomyelination phenotypes in multiple regions of the cerebrum and spinal cord reflecting a subset of clinical features found in patients. In contrast, the gross motor defects and cerebellar hypomyelination that are common features of severely affected patients are absent in the mice, suggesting a relatively mild form of the disease in this conditional model. Our results show that disease pathogenesis in the mice involves defects that reduce both the number of mature myelinating oligodendrocytes and the ability of these cells to produce a myelin sheath of normal thickness. Our findings uncover a critical role for Olig2 expressing cells in disease pathogenesis, specifically in the development and/or survival of oligodendrocytes as well as their function in myelination.

## POLR3-related leukodystrophy: Defining novel phenotypes from very mild to extremely severe

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POLR3-related leukodystrophy (POLR3-HLD) is a rare inherited neurological disorder caused by biallelic pathogenic variants in the genes *POLR3A, POLR3B, POLR1C,* and *POLR3K*, which encode for subunits of the enzyme RNA polymerase III. This disorder is typically associated with lack of proper myelin formation in the brain (hypomyelination), dental abnormalities (hypodontia), and endocrine abnormalities (hypogonadotropic hypogonadism). In this study, we define an expanded phenotypic spectrum of severity for POLR3-HLD through presentation of clinical, radiological, and molecular features of patients with very mild or extremely severe phenotypes. In addition, functional and neuropathological studies were performed on one patient with a severe phenotype. On the mild end of the spectrum, three patients homozygous for the same *POLR3B* variant (c.1568T>A; p.V523E) were diagnosed incidentally in adolescence or adulthood, with milder clinical and radiological characteristics compared to typical POLR3-HLD. On the severe end of the spectrum, six patients presented between ages 1-3 months with failure to thrive, severe dysphagia, and developmental delay, and four died before age 3 years. MRI and neuropathological studies revealed atypical characteristics, including basal ganglia and thalami abnormalities, with pathological evidence of neuronal loss in the putamen and caudate. Each patient harboured compound heterozygous variants in *POLR3A*, including a premature stop variant and a specific splice variant (c.1771-7C>G), which was determined to produce two aberrant transcripts, with detection of some wildtype transcript. Immunoblotting confirmed significantly decreased wildtype POLR3A protein levels in patient brain tissue samples compared to control. These findings demonstrate the extreme variability of POLR3-HLD disease severity, and provide insight into the pathophysiological complexity associated with variants in POLR3 subunits.

Perrier S et al. Expanding the phenotypic and molecular spectrum of RNA polymerase III-related leukodystrophy. *Neurology Genetics* 2020, 6 (3): e425.

# Session 17, Chair: David Schneider

**Elaine Sanij** - "Inhibition of RNA Polymerase I Transcription Activates the DNA Damage Response and Demonstrates Therapeutic Efficacy in Ovarian Cancer"

**Paul Trainor** - "Ribosomopathies: congenital disorders of craniofacial and peripheral nervous system development and the potential for their prevention"

**Steven Zheng** - "SOD1 Regulates Ribosome Biogenesis in KRAS Mutant Non-Small Cell Lung Cancer"

**Katherine Hannan** - "Development of 2nd generation RNA Polymerase I inhibitors for cancer therapy"

## Inhibition of RNA Polymerase I Transcription Activates the DNA Damage Response and Demonstrates Therapeutic Efficacy in Ovarian Cancer

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### Abstract

The first-in-class drug CX-5461 inhibits RNA polymerase I (Pol I) transcription of ribosomal RNA (rRNA) genes and has promising clinical activity in Phase I trials in patients with haematological malignancies and solid cancers. We have recently shown that CX-5461 has a significant therapeutic benefit *in vivo* in a cisplatin- and PARP inhibitors-resistant patient derived xenograft (PDX) model of high grade serous ovarian cancer (HGSOC) (Sanij et al., Nature Communication 2020). Our data demonstrate CX-5461 and PARPi exhibit different spectrum of cytotoxicity due to their distinct modes of action in inducing DNA damage response (DDR). CX-5461 activates the DDR at the rRNA genes within the nucleoli leading to global replication stress involving MRE11-dependent degradation of DNA replication forks. Importantly, CX-5461 exhibits efficacy in patient-derived HGSOC cells with reduced sensitivity to PARP inhibitors involving replication fork protection, a common mechanism of resistance to chemotherapy and PARPi. CX-5461 also co-operates with PARPi in exacerbating replication stress and enhances therapeutic efficacy against BRCA2-mutated HGSOC-PDX *in vivo*.

Further, we have identified CX-5461-sensitivity gene expression signatures in primary and relapsed HGSOC suggesting CX-5461 is a promising therapy for the treatment of ovarian cancer. Indeed, our recent work has demonstrated that CX-5461 exhibits powerful synthetic lethal interactions with various DNA repair pathways revealing the power of activating nucleolar DDR in cancer therapy (Yan et al., BJC 2020).

**Sanij E**.*,#, Hannan K. M.*, et al. CX-5461 activates the DNA damage response and demonstrates therapeutic efficacy in high-grade serous ovarian cancer. **Nature Communications (2020)** (1):2641. *co-first author, **#** co-corresponding authors

Yan S., et al., **Sanij E.** #, Pearson R. B. # and Chan K#. The RNA polymerase I transcription inhibitor CX-5461 cooperates with topoisomerase 1 inhibition by enhancing the DNA damage response in homologous recombination-proficient high-grade serous ovarian cancer. **British Journal of Cancer (2021)** 124, 616–627.

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**Ribosomopathies: congenital disorders of craniofacial and peripheral nervous system development and the potential for their prevention**

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Congenital disorders affect 1% of births, and craniofacial and gastrointestinal anomalies manifest quite frequently. Most of the bone and cartilage in the head and face, together with the peripheral nervous system, are derived from multipotent, migratory, neural crest cells. Craniofacial and gastrointestinal anomalies are therefore typically associated with defects in neural crest cell development. Recently, we discovered that rDNA transcription, which is a rate limiting step in ribosome biogenesis, plays an essential role in neural crest cell proliferation and survival. Mutations in genes that encode subunits of RNA Polymerase I, or accessory factors, lead to deficiencies in rDNA transcription and ribosome biogenesis, which results in neural crest cell death, and consequently cranioskeletal and peripheral nervous system defects. Why disruptions in global processes differentially affect distinct tissues during development and in the pathogenesis of ribosomopathy disorders remains unknown. Through lineage tracing, transcriptomic, expression, and translation analyses, we discovered that neural crest cells exhibit elevated levels of rRNA and protein synthesis compared to surrounding cells. This is necessary to meet their proliferation, growth and metabolic needs, but also renders neural crest cells particularly sensitive to disruptions in rDNA transcription and ribosome biogenesis via an Rpl5/11-Mdm2-p53 pathway. Consistent with this model, inhibition of p53 and cell death can prevent the pathogenesis of ribosomopathies. Our novel work therefore demonstrates the dynamic tissue-specific regulation and requirement for rRNA transcription during development but can also mechanistically account for the tissue-specific threshold sensitivities to perturbation of rDNA transcription in the pathogenesis of ribosomopathies. This work is supported by the Stowers Institute for Medical Research.

**SOD1 Regulates Ribosome Biogenesis in KRAS Mutant Non-Small Cell Lung Cancer**

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SOD1 is commonly known as the major cytoplasmic superoxide dismutase that are responsible for removal of superoxide free radicals. Mutations in SOD1 can cause amyotrophic lateral sclerosis (ALS), a motor neuron disease, and premature aging. Recent studies also show that SOD1 is overexpressed in non-small cell lung cancer (NSCLC) and is a promising anticancer target. However, the role of SOD1 in cancer is not fully understood. Herein we describe the generation of an inducible *Sod1* knockout in a KRAS-driven NSCLC mouse model. *Sod1* knockout markedly reduces tumor burden in vivo and blocks growth of KRAS mutant NSCLC cells in vitro. Surprisingly, SOD1 is found to be enriched in the nucleus and notably in the nucleolus of human and mouse NSCLC cells. The nuclear and nucleolar, not cytoplasmic, form of SOD1 is essential for lung cancer cell proliferation. Moreover, SOD1 is essential for biogenesis and nuclear export of ribosomes. Collectively, our study unexpectedly reveals a novel nuclear SOD1 function essential for ribosome biogenesis and proliferation in KRAS-driven lung cancer.

Wang XW, Zhang, H., Sapio, R., Yang J, Wong J, Guo J, Van Remmen H, Li H, White E, Liu C, Kiledjian M, Pestov, D. and Zheng X.F. SOD1 Regulates Ribosome Biogenesis in KRAS Mutant Non-Small Cell Lung Cancers. ***Nature Communications*** 2021, in press.

**Development of 2nd generation RNA Polymerase I inhibitors for cancer therapy.**

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RNA polymerase I (Pol I) transcription of ribosomal RNA (rRNA) genes (rDNA) is tightly-regulated downstream of oncogenic pathways and its dysregulation is a common feature in cancer. Our pioneering studies demonstrated great promise targeting rRNA as a cancer therapy using the 1st generation Pol I transcription inhibitor CX-5461. However, our latest research identified an off-target effect for CX-5461 associated with DNA damage signalling response (DDR) pathways. We hypothesise CX-5461's off-target activity contributed to the dose limiting toxicity (palmar-plantar erythrodysesthesia) reported in the clinical trial, which affirms the imperative for the generation of more selective inhibitors.

Thus, in collaboration with Pimera, we developed a selective 2nd-generation Pol I inhibitor, PMR-116, with improved selectivity, toxicology, tissue distribution and efficacy compared to CX-5461. Our ADME/DMPK analysis demonstrated that PMR-116: i) is orally available; ii) penetrates the blood brain barrier; iii) has excellent drug-like properties such as low activity in inhibiting or inducing members of the CYP450 family, low potential for unwanted drug-drug interactions, no significant effect on cardiac ion channels (>10μM); and iv) does not induce a DDR.

PMR-116 has a spectrum of activity across a variety of cancer cell lines on viability (50% growth inhibition: $GI_{50}$~280nM) with minimal effects on normal cells. PMR-116 also has significant efficacy in numerous *in vivo* cancer models including AML and prostate cancer (PCa). For example, in the Hi-MYC PCa mouse model dosing once weekly for 4 weeks decreased the incidence of invasive lesions by ~85% compared to vehicle and reverted glands to patterns of low-grade intraepithelial neoplasia. While in some patient-derived xenografts lines established from multidrug-resistant, metastatic PCa PMR-116 treatment decreased tumour volume, with a complete response observed in one line (tumour volume decreased by ~90% compared to baseline).

Based on our work a multicentre open-label multiple ascending dose study to assess the safety and tolerability of PMR-116 in patients with advanced solid tumours of any cancer type has commenced (Jan 6th 2021, lead oncologist Prof. Desai, Peter MacCallum Cancer Centre, Australia -ACTRN12620001146987).

# Session 18, Chair: Jennifer Gerton

**Steve Bell** - "Chromosome Archae-tecture"

**Carson J. Bryant** - "High-throughput global analysis of miRNA drivers of ribosome biogenesis"

**Olivier Gadal** - "RNA polymerase I mutant affect ribosomal RNA processing and impact ribosomal DNA stability"

**Brian McStay** - "The formation and internal organisation of human nucleoli, revealed one NOR at a time"

**Chromosome Archae-tecture**

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Over the last 25 years, steady progress has been made in advancing our understanding of the archaeal core transcription machinery and its regulation by gene-specific transcription factors. However, comparatively little is known about the nature and organization of the true *in vivo* substrate for gene expression – the archaeal nucleoid. To address this deficiency in our knowledge, we have exploited chromosome conformation capture approaches to interrogate the form and function of the chromosomes of hyperthermophilic archaea of the genus *Sulfolobus*.
We have found that multiple levels of organization structure the chromosomes. First, as in metazoa, chromosomes are organized into A and B-type compartments. Second, smaller domains are found throughout the chromosomes, similar in scale and number to "Chromosome Interaction Domains" first described in bacteria. Third, we observe direct apposition of defined loci in the form of loop structures. These latter loop structures have some important implications for coordination of ribosome biogenesis. Dissecting the rules governing this multi-scale organization, we provide evidence for key roles for transcription and its interplay with a novel SMC-superfamily protein that we term coalescin.

N. Takemata and S.D. Bell (2021) "Multi-scale architecture of archaeal chromosomes" Molecular Cell, 81, 473-487

N. Takemata, R. Samson and S.D. Bell (2019) "Physical and functional compartmentalization of archaeal chromosomes". Cell, 179, 165-179

**High-throughput global analysis of miRNA drivers of ribosome biogenesis**

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Ribosome biogenesis (RB) is the complex, coordinated, essential process that generates mature ribosomal subunits and ultimately enables all protein synthesis in the nucleolus of eukaryotic cells. While many known RB regulators are proteins or small nucleolar ribonucleoproteins, whether microRNAs (miRNAs) are a novel modulatory layer controlling ribosome production in mammalian systems is an open question. To globally identify miRNA regulators of ribosome biogenesis, we have conducted a high-throughput, high-content screen in human tissue culture cells for nucleolar number and function using a library of 2603 miRNA mimics in triplicate. Our screen has identified 64 miRNAs that, when upregulated, significantly decrease nucleolar number, and 9 miRNAs that significantly increase nucleolar number. Hits were selected using cutoffs for both mean percent effect versus a control for each nucleolar number phenotype, and for effect size and reproducibility as measured by strictly standardized mean difference (SSMD). For the one-nucleolus hits, analysis of validated miRNA targets using TarBase 8 revealed that the hits were enriched for targets that are either nucleolar or are known to change nucleolar number upon knockdown. We investigated the extent to which RNA polymerase 1 (RNAP1) activity was affected by each miRNA hit using a novel high-throughput assay for transcription in the nucleolus that employs 5-ethynyl uridine, click chemistry, and immunofluorescence. Strikingly, we find that 69% of the miRNA hits decrease the nucleolar signal by at least 50% relative to the positive control, the large subunit of RNAP1, siPOLR1A, suggesting a role in pre-rRNA transcription. Further work will elucidate the regulatory role of these miRNA hits in pre-rRNA processing and global protein synthesis. Our results systematically illuminate miRNAs as an important conduit between RB defects and disease pathogenesis in mammalian cells for the first time.

**RNA polymerase I mutant affect ribosomal RNA processing and impact ribosomal DNA stability**

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Transcription is a major contributor to genome instability. Ribosomal RNA genes (rDNA) locus is made of head-to-tail repeat of the most actively transcribed genes of the genome. Massive rRNA production depends on RNA polymerase I (Pol I), and is co-transcriptionally assembled with early ribosomal assembly factors in yeast nucleolus. In *Saccharomyces cerevisiae*, mutant form of Pol I bearing a fusion of transcription factor Rrn3 with Pol I subunit Rpa43 (CARA-Pol I) has been described previously (Laferté et al. 2006). Here, we show that CARA-Pol I allele resulted in rRNA processing defect associated with rDNA genomic instability. CARA-Pol I does not over-produce of rRNA, but a fraction of the primary transcript escape processing steps and accumulates. CARA-Pol I is synthetic lethal with mutants affecting rDNA condensation including monopolin mutants lrs4 or csm1. CARA-Pol I strongly impacts rDNA organization as shown by direct visualization of altered rDNA morphology in living cells and increased rDNA copy number variation. Reduced rDNA copy number, or rDNA removed from chromosome suppress lethality, showing that chromosome segregation defect is caused by genomic rDNA. We propose that rRNA escaping processing, impacts rDNA stability via R-loop formation .

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**The formation and internal organisation of human nucleoli, revealed one NOR at a time**

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Nucleolar organiser regions (NORs) comprising ribosomal gene (rDNA) arrays are located on the p-arms of the five human acrocentric chromosomes, HSA13, 14, 15, 21 and 22. We have recently shown that NORs are active by default. Within karyotypically normal primary and hTERT-immortalised human cell lines, all NORs with detectable levels of rDNA are active (van Sluis et al. 2020). Only in cancer lines do we find evidence for silent NORs. We further revealed that nucleolar association of acrocentric p-arms can occur independently of rDNA content, suggesting that sequences elsewhere on these chromosome arms drive nucleolar association.
We have recently devised a methodology for genetic manipulation of individual NORs that allows us to explore the internal organisation of nucleoli (Mangan and McStay 2021). Efficient 'scarless' genome editing of rDNA repeats is achieved on 'poised' human NORs held within mono-chromosomal cell-hybrids. Subsequent transfer to human cells introduces 'active' NORs yielding readily discernible functional customised ribosomes. We reveal that ribosome biogenesis occurs entirely within constrained territories, tethered to individual NORs inside a larger nucleolus.
Using this methodology we have also been able to precisely delete rDNA arrays from individual acrocentric chromosomes and demonstrate that the resulting chromosomes retain nucleolar association capability when reintroduced into human cells. These results compel us to revaluate so called "nucleolar fusion".

Mangan H, McStay B. 2021. Human nucleoli comprise multiple constrained territories, tethered to individual chromosomes. *Genes Dev*. **35:** 449-469

van Sluis M, van Vuuren C, Mangan H, McStay B. 2020. NORs on human acrocentric chromosome p-arms are active by default and can associate with nucleoli independently of rDNA. *Proc Natl Acad Sci U S A* **117**: 10368-10377.

# Session 19, Chair: Achim Griesenbeck

**Abigail Huffines** - "Investigating the regulation of RNA polymerase I by the transcriptional activator Hmo1"

**Soma Dash** - "Novel roles for RNA Polymerase I and associated factors in neural crest cell colonization of the gut and in the pathogenesis of Hirschsprung disease"

**Duy Khanh Phung** - "Structure, function and evolution of archaeal NusA paralogues"

**Kristin Scott** - "The Transcriptome-wide Distribution of Methyl-5 Cytosine in the Hyperthermophilic Archaeon, Thermococcus kodakarensis"

**Craig Marshall** - "Structural requirements for Eta-mediated archaeal transcription termination"

**Investigating the regulation of RNA polymerase I by the transcriptional activator Hmo1.**

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RNA polymerase I (Pol I) transcribes ribosomal DNA (rDNA) into ribosomal RNA (rRNA), which accounts for approximately 80% of the total RNA in the cell and, along with ribosomal proteins, is assembled into the mature ribosome. Transcription by Pol I is thought to be the rate-limiting step of ribosome biogenesis, which is upregulated during cancer. Therefore, Pol I has been identified as a potential target for cancer therapeutics. To develop effective new therapeutics, the characterization of transcription factors that regulate Pol I is critical. One such potential regulating factor in *Saccharomyces cerevisiae* (yeast) is high mobility group protein 1 (Hmo1), which is thought to be the functional analog of the human protein, upstream binding factor (UBF). Hmo1 has been proposed to be a transcriptional activator, however, its mechanism is still unknown and its regulation of Pol I has not been well-defined. Recently, it was determined that in Pol II-transcribed genes, Hmo1 may coordinate with topoisomerases, especially DNA topoisomerase 2 (Top2), to help regulate the topological landscape of DNA during transcription. Additionally, other groups have characterized yeast mutants containing either deletions in *HMO1* or *TOP2.* Both of these mutants displayed severe growth defects and a decrease in overall rRNA synthesis. Based on these previous insights, we hypothesize that Hmo1 may play a conserved role in transcription by Pols I and II as a mediator of the template DNA topology through its coordination with topoisomerases. To investigate this, we used a technique called native elongating transcript sequencing (NET-seq) to analyze the global effect of Hmo1 on transcription at single nucleotide resolution *in vivo.* Our NET-seq results demonstrate that in *hmo1*$\Delta$ yeast, there is a build-up of Pol I at the 3' end of the gene that is not present in wild-type (WT) yeast. We propose that this is due to unresolved topological constraints at the 3' end of the rDNA in *hmo1*$\Delta$ yeast. The findings from this study suggest that Hmo1 promotes efficient rRNA synthesis *in vivo* and identifies a novel role for this factor in transcription by Pol I.

**Novel roles for RNA Polymerase I and associated factors in neural crest cell colonization of the gut and in the pathogenesis of Hirschsprung disease**

Soma Dash*[1], Stephen Shannon[1], Kristin E.N. Watt[1], Karla Terrazas-Falcon[1], Paul A. Trainor[1,2]

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rRNA transcription by RNA Polymerase I and ribosome biogenesis are essential for all cell growth and survival. However, disruption in these processes result in tissue specific defects called ribosomopathies. Here we present novel data that Hirschsprung disease, a congenital disorder that occurs with an incidence of 1:5000 live human births and which is characterized by the absence of ganglia in variable regions of the gastrointestinal tract, can occur as a result of deficient rRNA transcription and impaired ribosome biogenesis. The ganglia in the enteric nervous system are derived from a multipotent, migratory population of cells called neural crest cells that arise from neuroepithelium during neurulation. Neural crest cell-specific loss of function mutations in RNA Polymerase I subunits, *Polr1a* and *Polr1c* and associated factor, *Tcof1* results in total intestinal aganglionosis, the most severe form of Hirschsprung disease in both zebrafish and mouse models. We are therefore exploring the function of Polr1a, Polr1c and Tcof1 in regulating neural crest cell development during enteric nervous system formation and testing avenues for preventing the pathogenesis of Hirschsprung disease. This work is supported by the Stowers Institute for Medical Research and Postdoctoral Fellowship from American Association of Anatomy.

**Structure, function and evolution of archaeal NusA paralogues**

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Transcription is one of the most fundamental processes in biology with an intriguing conservation of factors and mechanisms - a mosaic of vertical descent and convergent evolution. The core of all double-psi beta barrel (DPBB) RNA polymerases (RNAP) is universally conserved. Most of the basal transcription factors in archaea are closely related to their eukaryotic homologs, with the exception of archaeal NusA that is ubiquitously present in all prokaryotes but has no apparent homolog in eukaryotes. *E. coli* NusA is an essential elongation factor that modulates the processivity and pausing of RNAP, stabilises RNA-hairpin mediated RNAP pauses, is part of antitermination complexes, and facilitates rRNA folding and maturation. Archaeal NusA factors have a reduced domain architecture encompassing two RNA-binding KH domains only, missing the S1, the RNAP-interaction and regulatory domains characteristic of bacterial NusA. Many archaea encode two highly divergent NusA paralogues (NusA1 and -2). Archaeal NusA factors are essential for cell viability but their function remains unknown.
Here we focus on a multidisciplinary characterisation of *Sulfolobus solfataricus* NusA1 and -2. ChIP-seq profiling shows that NusA1 is recruited proximal to gene promoters – rather than behaving like a *bona fide* elongation factor mapping within transcription units like in bacteria. NusA1 interacts with archaeal preinitiation complexes (PIC) in EMSAs and moderately inhibits RNAP in promoter-directed in vitro transcription assays. More importantly, biochemical co-sedimentation experiments show that NusA1 interacts with small ribosomal subunit, altogether suggesting a role in the recruitment of the lead ribosome to the RNAP proximal to the gene promoter during the coupling of transcription and translation. In contrast, the divergent NusA2 paralogue could not be ChIP'ed to genomic DNA, and did not associate with any higher order complexes, which suggests a different role from NusA1. We solved the X-ray structure of NusA2 at atomic resolution, it confirms the presence of the ZF domain and reveals a stunning relationship to the eukaryotic ribosomal protein S7e; both form a highly divergent but monophyletic group. Yet, our biochemical analyses do not support the notion that NusA2 is stably incorporated into the majority of archaeal ribosomes under our experimental conditions but NusA2 may represent a 'specialised' ribosomal protein subject to regulation.
In summary, the work presented here provides links between NusA variants, and the transcription and translation machineries in archaea.

**The Transcriptome-wide Distribution of Methyl-5 Cytosine in the Hyperthermophilic Archaeon, *Thermococcus kodakarensis***

**Kristin A. Scott[*,1,2], Hallie Febvre[2], Rebecca Zimmerle[2], Brett Burkhart[2], F. Brett Rob[3], Ryan Fuchs[3], Thomas Santangelo[1,2]**

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Methyl-5 cytosine (m5C) has been shown to impact the structure and function of rRNA and tRNA, however, the transcriptome-wide distribution of m5C has not been fully elucidated. *Thermococcus kodakarensis*, a hyperthermophilic archaeon, is an ideal model to investigate RNA modification, as modified residues are likely critical for RNA stability at high temperatures. Sodium bisulfite treatment of total RNA followed by a deep-sequencing approach revealed 641 high-confidence m5C sites, with 68% of detected sites (438 sites) present in mRNA. In mRNA, m5C persists with a ~20% frequency within a modestly conserved GGGC motif. Bisulfite-sequencing of 13 unique strains, each deleted for a single RNA methyltransferase, revealed between dozens to hundreds of differentially methylated sites. These results quantitatively define the m5C landscape in *T. kodakarensis* and provide a foundation to support future studies into how m5C modification impact transcript behavior.

**Structural requirements for Eta-mediated archaeal transcription termination**

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Transcription must be properly regulated both temporally and spatially to ensure proper and dynamic gene expression. Such regulation is achieved throughout the transcription cycle of initiation, elongation, and termination. A host of transcription factors can promote or inhibit efficient initiation, and many have been shown to both increase and decrease rates of elongation- all through interactions with RNA polymerase (RNAP). Inducing termination of stable active transcription elongation complexes (TECs), effectively reducing the elongation rate to zero, is a more difficult task requiring collapse of the transcription bubble and release of both RNAP and the RNA transcript from DNA. Intrinsic termination events, usually dependent on intrinsic U-rich sequences at the end of coding regions of DNA and thus the resultant RNA transcript, are documented across Domains, but only a handful of protein transcription termination factors are known. We previously identified the first archaeal factors capable of disrupting the TEC. Eta, a well conserved superfamily II (SF2) helicase, appears to terminate transcription of stalled transcription elongation complexes (TECs). FttA, a CPSF73 homolog conserved throughout the Domain, appears to be responsible for global transcription termination after the uncoupling of transcription and translation.

With hopes of delineating SF2 helicase mechanisms leading to susceptibility of extremely stable TECs, we recently conducted a structure-function relationship study of Eta employing select mutations derived from a crystallographic structure. Several mutant forms of Eta were mutagenized, expressed, isolated, and ultimately assayed for any defects in motor or termination activities. We identified multiple different point mutations which significantly affected motor activity, termination activity, or both, and used these insights to guide a structural model of the Eta-TEC transcription termination complex. Our work further explores ‘general’ and ‘specialized’ factor-mediated termination activities in Archaea, and hopes to provide insight into exact mechanisms of transcription termination while adding to the field of SF2 helicases and exploring the evolutionary roots of eukaryotic transcription termination.

# Session 20, Chair: Linda van Dyk

**Nazif Alic** - "Odd Pols in fruit fly ageing"

**Ellen Busschers** - "A novel role for MAF1 and RNA pol III-dependent transcription in osteoblast differentiation and bone biology"

**Damian Graczyk** - "Manipulation of MAF1 levels affects pro-inflammatory functions of mouse macrophages"

**Odd Pols in fruit fly ageing**

Danny Filer[1], Guillermo Martınez Corrales[1], Sara Javidnia[1], Abbie Rogan[1], Mengjia Li[1], Katharina C. Wenz[1], Stefanie Mueller[2], Susan J. Broughton[3], Karoline Kuchenbaecher[2] and Nazif Alic[1]*
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Ageing and the associated functional decline are of growing importance to contemporary societies. Work in model organisms has revealed that ageing itself can be modulated and understanding the underlying molecular mechanisms presents an opportunity to devise interventions to improve health and wellbeing in older humans. Several signalling pathways that accelerate the rate of ageing, such as insulin/IGF and Target of Rapamycin signalling pathways, also stimulate the activity of RNA polymerase I and III (Pol I and III). This prompted us to examine the role of Odd Pols in ageing. We find that both Pol I and Pol III activity in the adult fruit fly limit the animal's health and survival in old age. The two Pols act on ageing predominantly from the fruit fly intestine, specifically from the intestinal stem cells but Pol I activity in the terminally differentiated enterocytes also curtails lifespan. We have recently explored the potential relevance of Odd Pols to human ageing. Using human population genetic analyses, we find evidence that expression of Pol I or Pol III-specific subunits is associated with human longevity. These findings implicate Odd Pols in human ageing.

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**A novel role for MAF1 and RNA pol III-dependent transcription in osteoblast differentiation and bone biology.**

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Our previous work revealed that MAF1, a repressor of RNA pol III-dependent transcription, plays a role in mesoderm formation as well as the differentiation of adipocytes. We hypothesized that MAF1 may also play a role in the cellular differentiation of other mesenchymal cell types. Increased MAF1 expression in ST2 cells, a stromal cell line, produced an increase in the differentiation of these cells into osteoblasts, whereas MAF1 knockdown impaired osteoblast differentiation. To determine the role of MAF1 *in vivo* in bone biology, MAF1 was overexpressed in mouse long bones in a PRX1-CRE-dependent manner. This resulted in bones with higher bone volume and density. Collectively, these results indicate that MAF1 stimulates osteoblastogenesis *in vitro* and it promotes bone volume and density *in vivo*. We further determined if repressing RNA pol III transcription using other approaches would have a similar effect on osteoblastogenesis. Surprisingly, inhibition of RNA pol III-dependent transcription by either chemical inhibition of RNA pol III using ML-60218, or by BRF1 knockdown, produced a significant decrease in osteoblast differentiation. Thus, while MAF1 stimulates osteoblast differentiation, repression of RNA pol III-dependent transcription by other approaches results in a decrease in differentiation. To determine the basis for these opposing effects by MAF1 compared with BRF1 knockdown or ML-60218 treatment, changes in gene expression were examined by RNA-seq, prior to and during, the differentiation process. Altering RNA pol III-dependent transcription by changes in MAF1, BRF1 or ML-60218 treatment produced distinct gene expression changes with limited overlap amongst these different conditions. Changes produced by MAF1 overexpression were significantly enriched for changes in extracellular matrix regulation, consistent with a role for MAF1 in osteoblast development. These results suggest that different approaches to repress RNA pol III-dependent transcription do not result in the same gene expression changes. Furthermore, MAF1 produces distinct gene expression changes from those produced by changes in BRF1 expression or RNA pol III inhibition that may account for its opposing effects on the differentiation process. Overall, our results demonstrate a novel role for MAF1 and RNA pol III-dependent transcription in osteoblast differentiation and bone biology. Together with our previous studies on adipogenesis, the current work provides further to support for the ability of RNA pol III-dependent transcription to impact cell fate determination.

## Manipulation of MAF1 levels affects pro-inflammatory functions of mouse macrophages

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Macrophages are phagocytic cells of the innate immune system. They have many biological functions, such as antigen presentation, target cell cytotoxicity, phagocytosis and regulation of inflammation. Activation of macrophages with lipopolysaccharides (LPS), a major component of the outer membrane of most Gram-negative bacteria, induces rapid transcriptional changes of several hundred genes. LPS are sensed by the Toll-like receptor 4 (TLR4) for induction of various signalling pathways and activation of several transcription factors to establish a broad anti-microbial, pro-inflammatory programme. The biosynthetic burst associated with the production of new proteins requires efficient translational machinery, which in turn relies on many elements including transfer RNA (tRNA). tRNAs are produced by RNA polymerase III (Pol III), and tRNA synthesis is increased in macrophages stimulated with LPS.

Here we show that MAF1, an evolutionary conserved negative Pol III regulator, affects macrophage function by limiting the production of specific pro-inflammatory cytokines and downregulates the expression of interferon-stimulated genes. MAF1 also inhibits phagocytosis. Mechanistically, MAF1 hampers TRIF-dependent TLR4 signalling and impinges on IRF3 and late NF-κB activation. This results in lower IFNβ production, weaker activation of JAK/STAT signalling pathway and, in consequence, weaker activation of interferon-stimulated gene expression. Overall, the data show a previously unanticipated role of MAF1 in the regulation of interferon response in macrophages.

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