Wojciech Siwek

Epigenetic mechanisms in maintenance of active transcriptional states

Summary of Professional Accomplishments

International Center for Cancer Vaccine Science, University of Gdańsk

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Gdańsk, 2023

1. Name

Wojciech Siwek

2. Diplomas and degrees

2/12/2014 **PhD in Biochemistry (with distinctions)**,
International Institute of Molecular and Cell Biology;
Institute of Biochemistry and Biophysics, Polish Academy of Sciences,
mentor: Matthias Bochtler

title: "The mechanism of action of N6-methyladenine dependent restriction endonuclease R.DpnI",

6/2/2011 **Methods in Analytical Chemistry**, postgraduate studies,
University of Warsaw, PL

3/7/2007 **MSc in Biotechnology**, University of Warsaw;
Institute of Biochemistry and Biophysics, Polish Academy of Sciences

13/1/2006 **BSc in Biotechnology**, University of Warsaw, PL

3. Professional experience

2023 – present **Assistant Professor**, International Centre for Cancer Vaccine Science, University of Gdańsk, PL

2021 - 2023 **Independent Research Fellow** (Marie Skłodowska-Curie fellow), Department of Molecular Biology, Massachusetts General Hospital; Department of Genetics, Harvard Medical School, Boston, USA, mentor: Bob Kingston

2020 - 2021	**Research Fellow**, Department of Molecular Biology, Massachusetts General Hospital; Department of Genetics, Harvard Medical School, Boston, USA, mentor: Bob Kingston
2019 - 2020	**Research Associate**, Department of Biochemistry, University of Oxford, UK, mentor: Lars Jansen
2015 - 2019	**Postdoctoral Fellow** (with a scholarship from the Fundação para a Ciência e Tecnologia, PT), Instituto Gulbenkian de Ciência, PT, mentor: Lars Jansen
2007 - 2014	**PhD Student**, International Institute of Molecular and Cell Biology, PL, mentor: Matthias Bochtler
2005 - 2007	**MSc Student**, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, mentor: Jacek Hennig
2006 - 2006	**Intern** (with a scholarship), Sainsbury Laboratory, John Innes Centre, Norwich, UK

4. Description of the achievement

4.1. Title of the scientific achievement

Epigenetic mechanisms in maintenance of active transcriptional states

4.2. List of publications constituting the scientific achievement

1. **Wojciech Siwek***, Sahar S.H. Tehrani, João F. Mata and Lars E.T. Jansen*. (2020) Activation of clustered IFNγ target genes drives cohesin-controlled transcriptional memory. Molecular Cell. doi.org/10.1016/j.molcel.2020.10.005
   ***Corresponding authors**

Impact Factor = 19.328 (Academic Accelerator, 2022-2023), Citations = 28 (Google Scholar, 20/09/23), Points awarded by the Ministry of Education and Science = 200 (2023)

My contribution comprised of conceptualization of the study as well as design and execution of the experiments. Specifically, I performed the bulk transcriptome measurements resulting in the discovery of a group of genes GBPs (Guanylate Binding Proteins) as new memory genes; validated the observation on RNA and protein levels as well as in primary cells; determined the duration of interferon gamma (IFNγ) transcriptional memory; characterized the stochastic nature of the phenomenon by single-cell RNA-seq (scRNA-seq), cytometry and FACS (Fluorescence-activated Cell Sorting); established that transcription is dispensable for memory maintenance by long-term measurements of RNA output and perturbations with a transcriptional inhibitor; showed that none of the previously proposed chromatin modifications are maintained on the memory genes for the length of transcriptional memory; determined that key memory genes reside in genomic clusters and discovered a novel regulatory function of the cohesin complex in establishment of IFNγ transcriptional memory. Moreover, I analyzed and curated the data, supervised the work of other co-authors, created all the figures and wrote the manuscript.

This work was highlighted by Science Immunology: "A cytokine to remember me by"[1] and covered by national media, Science in Poland (scienceinpoland.pap.pl): "How do cells memorize information? A new lead."[2]

2. Sahar S.H. Tehrani, Pawel Mikulski, Izma Abdul-Zani, João F. Mata, **Wojciech Siwek***, Lars E.T. Jansen*. (2023) STAT1 is required to establish but not maintain interferon-γ-induced transcriptional memory. EMBO J. doi.org/10.15252/embj.2022112259
   ***Corresponding authors**

   Impact Factor = 13.783 (Academic Accelerator, 2022-2023), Citations = 3 (Google Scholar, 20/09/23), Points awarded by the Ministry of Education and Science = 200 (2023)

   My contribution comprised of conceptualization and initiation of the study, design of the experiments, hiring and training of co-workers, data curation and analysis, work supervision and writing of the original draft of the publication.

3. **Wojciech Siwek*,** Mariluz Gómez-Rodríguez*, Daniel Sobral, Ivan R. Corrêa Jr and Lars E.T. Jansen. (2018) time-ChIP: a method to determine long-term locus-specific nucleosome inheritance. Methods in Molecular Biology. doi.org/10.1007/978-1-4939-8663-7_7 ***Equal contribution**

Impact Factor = 0.368 (Academic Accelerator, 2022-2023), Citations = 6 (Google Scholar, 20/09/23), Points awarded by the Ministry of Education and Science = 70 (2023)

My contribution comprised of performing the experiments, specifically the H3.3 time-ChIP coupled to high throughput sequencing, together with the intersection of data with specific histone mark domains (H3K9ac, H3K27ac, H3K9me3 and H3K27me3). Moreover, I established a detailed protocol for the method (which is part of the publication), benchmarked the approach to other existent techniques, created the figures and wrote the manuscript.

Statements of co-authors on individual contribution - Appendix 5.

4.3. Review of the scientific objective and results obtained in the presented publications and their potential application

4.3.1. Introduction

Epigenetics is a process that is rooted in developmental biology and describes a heritable phenotype resulting from changes in the cell without alterations in the DNA sequence[3]. Epigenetic phenomena play a crucial role in preserving patterns of gene expression as an organism grows and continues its development into adulthood. This means that cells employ epigenetic mechanisms to determine whether specific genes should be turned on or off, which is essential for constructing various tissues and enabling specialized functions within the body. Hence epigenetics is fundamental for multicellular life[4].

Maintenance of gene expression states is regulated by feedback loops. These can work by cis-acting elements – non-diffusible stretches of DNA in proximity of genes that they control; or trans-acting factors – diffusible regulatory proteins capable of DNA binding and transcriptional activation[5]. It is quite remarkable that cells can retain specific gene expression even after undergoing processes like reprogramming[6,7] or nuclear transplantation[8]. This

highlights the impressive resilience of these mechanisms. In other words, despite major changes or manipulations, the systems maintaining gene expression states remain surprisingly stable and reliable.

Chromatin is a protein-DNA complex, responsible for packaging of genetic material and is in the center of cis-acting feedback loops that control maintenance of gene silencing. Dedicated cellular machinery is employed to read and copy chromatin marks to maintain the silent state during the cell cycle – replication and mitosis[9]. Examples of such epigenetic mechanisms include DNA methylation of CpG islands[10], and histone methylation: H3K9me heterochromatin[11,12] and H3K27me facultative heterochromatin[13].

Transcription factor feedback loops, on the other hand, can maintain both silent and active gene expression states[14–16]. Within these loops, transcription factors not only regulate the expression of specific target genes but also their own. Once induced for the first time, a transcription factor activates transcription of its own gene and, in this way, maintains transcriptional programs indefinitely[17].

Strikingly, feedback loops involving transcription factors can sustain active states, but they are not always essential for this purpose. An example of such independent action is the trans-differentiation transcription factor – MyoD[18]. Expression of MyoD is sufficient to change fibroblast to myoblast cell fate but is not required for maintenance of muscle cell identity and marker gene expression, as shown by temporal depletion studies in a mouse model[19]. The finding implies that aside from transcription factor feedback loops, other, possibly cis-acting processes (like those operating during silencing) are involved in preserving active transcriptional states; and yet, surprisingly such mechanisms are not understood. This lack of knowlage is a result of the complex nature of transcription, which is a highly dynamic process involving many components[20]. Due to this reason the field of epigenetics is almost exclusively focused on the mechanisms responsible for sustained gene silencing.

The way to get access to novel, cis-acting processes controlling preservation of active states, is to uncouple transcription from the components that ensure its continuous operation, as it is naturally happening during sustained gene silencing. This, however, is difficult to achieve experimentally as the two processes are tightly linked. To accomplish such uncoupling and uncover new chromatin based epigenetic mechanisms regulating maintenance of active gene expression states, I am investigating a phenomenon present in the innate immune system: **trained immunity** *(Fig.1A).* During this process, also known as innate immune memory, cells exposed to a pathogen can initiate an innate immune response to fight off the threat. Once the infection is resolved, these cells remember the stress. Consequently, if they encounter an insult

again, they respond more robustly to it. Importantly, this is not part of the adaptive immune response as the effect can only last a few months and is not specific to a given microbe[21]. Organisms exposed to a certain class of pathogens show enhanced immunity to other infection agents. For example, treatment of mice with fungal-derived β-glucan gives rise to enhanced immunity against a bacterial pathogen – *Staphylococcus aureus*[22].

In my postdoc work, I have been studying trained immunity at the level of gene expression – an occurrence called **transcriptional memory** *(Fig.1B).* When cells are initially exposed to a specific signal or trigger, they become primed. Later, days after the initial exposure, when these primed cells are restimulated, they exhibit heightened rates of gene expression[23].

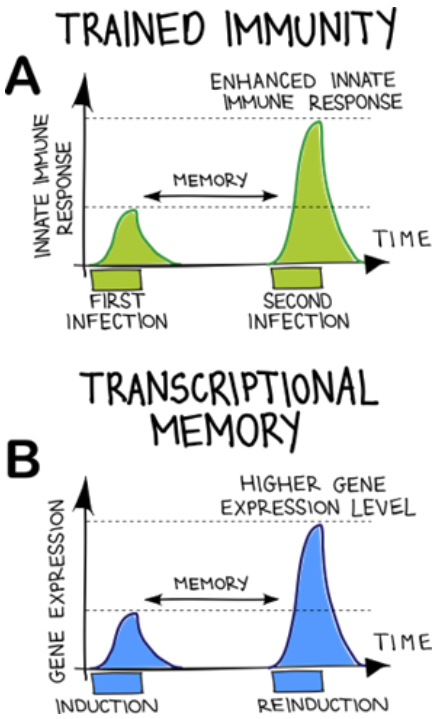

Figure 1. Trained immunity and transcriptional memory. ***(A)*** *Principle of trained immunity: an organism mounts an innate immune response to an infection and an enhanced reaction upon a second infection.* ***(B)*** *Principle of transcriptional memory: genes respond to a given signal and upon a second exposure to the same signal are expressed more strongly.*

The interferon response serves as a valuable model system to study this epigenetic process[24–28]. In my research, I have successfully developed a reliable procedure to investigate this aspect by utilizing interferon gamma (IFNγ) for stimulation[27]. By studying IFNγ transcriptional memory, I tapped into a unique opportunity to untangle the gene expression maintenance mechanism from the process of transcription in ways that were previously unexplored.

In this work, I aimed to discover novel *in cis* epigenetic mechanisms controlling maintenance of active gene expression states, by understanding cellular and molecular aspects of interferon-gamma (IFNγ) transcriptional memory; and develop a novel method to study this problem.

4.3.2. Detailed description of the publications included in the achievement

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Wojciech Siwek*, Sahar S.H. Tehrani, João F. Mata and Lars E.T. Jansen*. (2020) Activation of clustered IFNγ target genes drives cohesin-controlled transcriptional memory. Molecular Cell. doi.org/10.1016/j.molcel.2020.10.005

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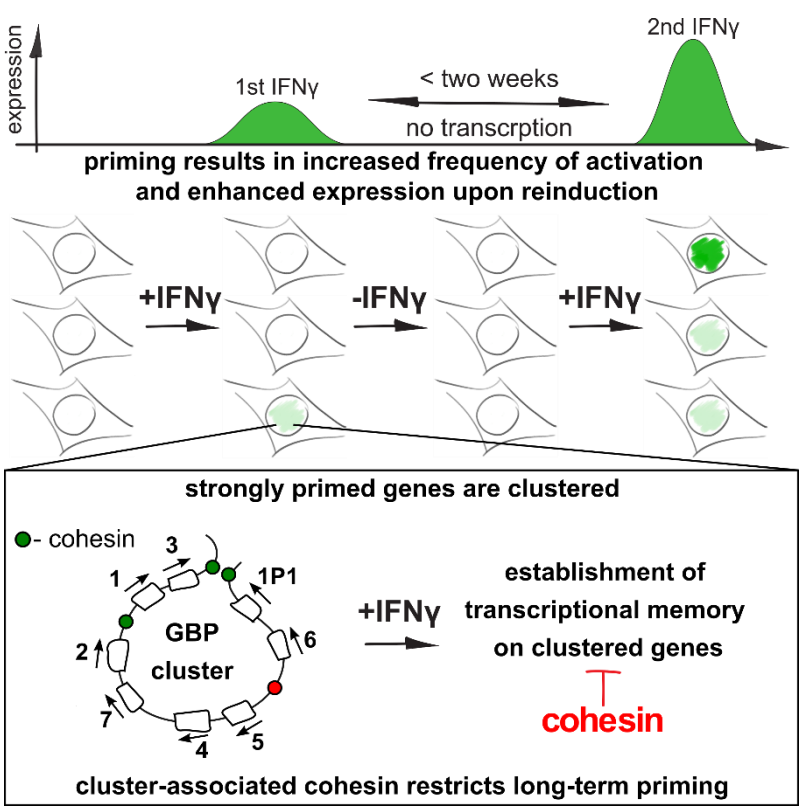

Figure 2. Activation of clustered IFNγ target genes drives cohesin-controlled transcriptional memory. *IFNγ transcriptional memory is maintained through up to 14 cell division cycles. No transcription is required for memory maintenance. Transcriptional memory is achieved via an increased probability of previously induced cells to engage in transcription. Memorized genes tend to reside in clusters for which memory initiation is restricted by cohesin.*

During IFNγ transcriptional memory cells exposed to the cytokine (primary activation) remember the experience and will respond to the second exposure (secondary activation) to the same stimulus more strongly. Importantly, previous work showed that IFNγ transcriptional memory is maintained in the absence of expression of the memory genes[24,25] (of note is that those measurements were only done shortly after removal of IFNγ). Therefore, in principle the system offers a unique opportunity to separate transcription from the maintenance component and hence discover new chromatin-based mechanisms regulating sustained gene expression.

Discoveries from other laboratories showed that an MHC class II gene, HLA-DRA has a strong IFNγ transcriptional memory phenotype in HeLa cells[24]. Further, a correlation was established that histone H3K4 dimethylation (H3K4me2), RNA PolII[24,25], H3.3 histone variant and histone H3K36 trimethylation (H3K36me3)[26] are retained on promoters of memory genes. Importantly, as for the transcriptional output, those measurements were only done shortly after removal of the cytokine. Aside from the chromatin features other factors were implicated in the process: Nup98 – a component of the nuclear pore[25], as well as the CDK8+ (positive) mediator complex[29].

Here we aimed to characterize IFNγ transcriptional memory in greater detail to establish the system as a model to study chromatin-based feedback loops regulating maintenance of active gene expression states and gain initial mechanistic insight into the phenomenon.

First, to expand the IFNγ transcriptional memory observation above the established model gene (HLA-DRA). We performed an RNA-seq, transcriptome measurement and discovered a new gene family GBPs (Guanylate Binding Proteins) showing strong transcriptional memory phenotype. GBPs are a group of factors that play a role in directly restricting intracellular pathogen replication[30], as well as promoting the inflammatory response[31]. Out of those, GBP5 displayed the strongest overall effect, stronger than the previously established model – HLA-DRA. We validated the GBP5 transcriptional memory observation by RT-qPCR and went further to show that the effect can also be measured on the protein level by western blot. Importantly, we discovered that IFNγ transcriptional memory is not restricted to cancer cells (HeLa) as primary fibroblasts also display IFNγ-mediated memory.

Previously, other laboratories had measured the memory effect for 48h after removal of the cytokine[24,25], however the exact duration of memory was never established. Given this gap in knowledge, we decided to determine the temporal extent of IFNγ transcriptional memory. We stimulated HeLa cells with IFNγ, removed the cytokine and allowed the cells to grow for several days. We next sampled the cells (post reinduction) for protein and RNA measurements. We discovered that memory lasts up to 14 days, an equivalent to many cycles of cell division. After that time cells go back to their original state, losing the primed condition. In essence, our research shed light on the fact that IFNγ-induced transcriptional memory can last for a significant period, but it eventually fades as the cells undergo more divisions.

Next, we characterized the IFNγ transcriptional memory effect on the cellular level by performing a single cell RNA-seq experiment. Our findings revealed that the memory phenomenon is a result of two combined factors: (1) increased GBP5 transcription – an upsurge

in the transcription of the GBP5 gene; and (2) increased participation of cells – more cells get involved in gene expression upon restimulation. Importantly, the second aspect has a much more substantial overall impact. Further, we consolidated this observation by engineering a cell line with a GFP cassette inserted into the GBP5 gene. With this tool and cytometry analysis, we confirmed that the major effect of the IFNγ transcriptional memory indeed comes from changing the probability of cells to engage in transcription after initial stimulation and not enhanced transcription of the GBP5 gene. Moreover, with the engineered cell line we performed FACS (Fluorescence-Activated Cell Sorting) tracking experiment and found that cells that in the initial stimulation engaged in transcription have a higher chance to express GBP5 upon restimulation. In essence, our investigation showcased that IFNγ transcriptional memory involves both increased gene transcription and a larger number of cells getting involved in gene expression upon restimulation. This enhanced participation is the major contributor to the phenomenon, and we validated this through specialized cell lines and tracking experiments.

We next decided to test if the process of transcription is required for maintenance of transcriptional memory. We wanted to explore whether even minimal levels of transcription could be adequate to keep genes in a poised state, ready for reactivation. To examine this, we conducted the following experiment. We first stimulated cells with IFNγ and then removed the cytokine. After several days, we collected the samples, without reinducing with IFNγ, and processed them for highly sensitive RT-qPCR analysis. We found that expression of memory genes (GBP5 and HLA-DRA) returns to baseline after removal of IFNγ. To further explore this idea and extend our observation to a broader context, we used an RNA PolII inhibitor (triptolide) to test if transcription of other non-memory genes might be important in memory maintenance – which is a hallmark of transcription factor feedback loops. We briefly treated cells with the inhibitor after stimulation with IFNγ. The goal was to see if blocking transcription, not just for memory genes but for other non-memory genes as well, impacted memory maintenance. We found that global transcription is not required for maintenance of IFNγ transcriptional memory, pointing to a chromatin-based mechanism of maintenance.

To determine which chromatin features may play a role in maintenance of transcriptional memory, we performed genome-wide analysis for accessibility (ATAC-seq) and occupancy of different factors and marks (ChIP-seq): RNA PolII, H3.3 histone variant and histone modifications: H3K27ac, H3K36me3, H3K79me2, H3K4me2. We determined that none of those molecular marks are maintained on the memory genes long-term after removal of IFNγ, with a notable exception of H3K4me2 detectable on memory genes 2 but not 7 days

after removal of the cytokine. Considering our discovery that memory can last up to 14 days, this makes those molecular signatures unlikely to play a key role in maintenance of transcriptional memory. This finding contradicts previous reports that had suggested otherwise[24–26].

After that, we decided to change our approach and took a closer look at the genomic positions of the memory genes. We found that the most strongly memorized genes reside in clusters, they are adjacent to each other in the genome. This raised the possibility that a single memory mechanism could regulate multiple genes. An example of a regulatory mechanism spanning multiple genes is the developmentally regulated globin gene cluster. A locus control region selecting expression of a specific globin gene dependent on the developmental stage[32]. To test if similar mechanism might be at play for our system, we decided to perturb the cohesin complex, known to be the key protein factor maintaining genome folding[33]. We used auxin-inducible degron[34] to globally remove cohesin, hence unfold the genome, after memory establishment. We found that this severe genomic perturbation has no effect on memory maintenance.

Next, we decided to see if genome folding plays a role in the establishment of IFNγ transcriptional memory. For that reason, we again used our auxin degron to remove cohesin but this time just before the first induction. We assayed the effect on memory with an RNA-seq measurement. Surprisingly, we observed enhanced memory for the clustered MHC class II and GBP memory genes. To further understand this observation, confirm that cohesin regulation is achieved by local sites and not some nonspecific effect of global cohesin removal, we performed a ChIP-seq experiment to detect genomic positions of cohesin in proximity of the GBP cluster. With this knowledge, we removed several cohesin sites from the vicinity of the GBPs by CRISPR-Cas9 excision and measured the effect on the GBP and MHC class II genes. We identified a single cohesin site in the GBP cluster that inhibits the establishment of IFNγ transcriptional memory but only locally at the GBP genes and not at the other memory cluster – MHC class II.

In summary, in this work we established a robust system, based on a new model memory gene – GBP5, to study IFNγ transcriptional memory and confirmed that the effect is not cancer specific. We characterized the phenomenon in detail: determined the length of memory and its stochastic nature. Moreover, we gained initial insights into the molecular mechanisms controlling initiation of the process *(Fig. 2).*

On top of that, we determined that IFNγ transcriptional memory is a specific locally controlled process. Two lines of evidence suggest this: (1) initiation of transcriptional memory

is not just dependent on the signaling pathway as only a small group of IFNγ responsive genes show memory, but a few thousand are induced by the cytokine; (2) perturbing cohesin binding sites (a protein complex responsible for genome folding) in proximity of GBPs leads to enhanced memory formation of these, but not the MHC II – other class of memory genes, located elsewhere in the genome.

Most importantly, we demonstrated that IFNγ transcriptional memory is not a transcription factor feedback loop as: (1) it is not permanent (2) no stable expression of transcription factors; or (3) sustained open chromatin (a hallmark of transcription factor binding) could be detected in primed cells; and finally (4) transcription is not required for memory maintenance.

Sahar S.H. Tehrani, Pawel Mikulski, Izma Abdul-Zani, João F. Mata, **Wojciech Siwek***, Lars E.T. Jansen*. (2023) STAT1 is required to establish but not maintain interferon-γ-induced transcriptional memory. EMBO J. doi.org/10.15252/embj.2022112259

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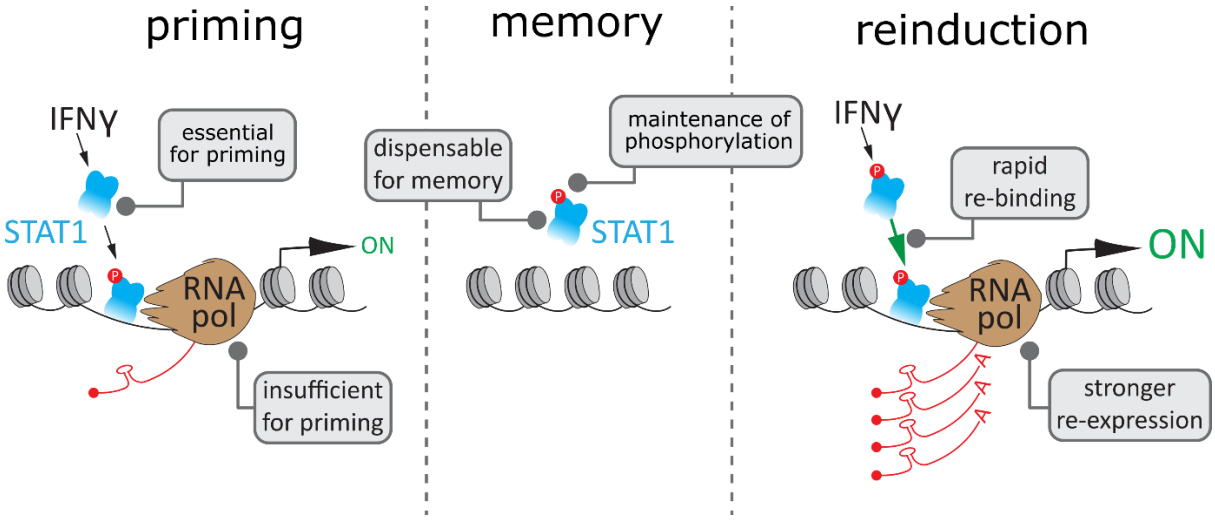

Figure 3. STAT1 is required to establish but not maintain IFNγ transcriptional memory. *Transcription is not sufficient to induce memory. STAT1 is essential to establish the primed state but dispensable for memory maintenance. STAT1 S727 phosphorylation is maintained during the primed state. IFNγ stimulation results in accelerated STAT1 and IRF1 recruitment to the memory genes in primed cells.*

The process that triggers initiation of IFNγ transcriptional memory is not understood. While the role of transcription factors in transcriptional memory has been established in plant[35,36] and yeast systems[29,37], such mechanisms have not been described for mammalian transcriptional memory.

Rich literature describes a pair of dedicated transcription factors (STAT1 and IRF1) working in a cascade fashion governing IFNγ signaling[38]. When IFNγ binds to its receptor, the first transcription factor that responds is STAT1. It becomes activated through phosphorylation. This is essentially the first step triggered by the cytokine's binding[38]. STAT1 activation then leads to the expression of a secondary transcription factor known as IRF1. Its involvement additionally contributes to the cellular response to IFNγ[39].

Here we used our established IFNγ transcriptional memory system to understand the general role of transcription and specific functions of key transcription factors families, STATs and IRFs in the initiation and maintenance of transcriptional memory.

As a first step, to test if the sole process of transcription of a target gene is sufficient for establishment of IFNγ transcriptional memory, we decided to bypass the IFNγ signaling pathway altogether by activating a memory gene with a synthetic biology tool. We used CRISPR-Cas9 Synergistic Activation Mediator (CRISPRa-SAM)[40] for this purpose. We established conditions to activate the GBP1 memory gene and discovered that transcription alone is not sufficient to initiate memory. This means that a system specific to the IFNγ signaling pathway is required for memory introduction.

To better understand how memory genes are controlled, we performed a time-course chromatin accessibility measurement (ATAC-seq) to determine the kinetic differences in promoter opening of memory genes in naïve and primed cells. As anticipated, we observed that chromatin around memory genes opened faster when cells were reinduced with the stimulus compared to their initial activation.

Next, to understand what potential transcription factors might play a role in memory, we screened multiple candidates from the STAT and IRF families by CRISPR-Cas9 mutagenesis (STAT1, STAT2, STAT3, STAT5B, IRF1, IRF9). We found STAT1 and IRF1 as key players in GBP5 gene activation. After that, we performed a CUT&RUN[41] kinetic experiment. This method allowed us to track the binding of STAT1 and IRF1 to the genes in both naïve and primed cells. This tracking revealed the timing of their recruitment to the memory genes throughout the process. We observed that during the reinduction stage, STAT1 and IRF1 were recruited to the memory genes at a faster pace compared to the initial activation.

Further, we focused our attention on STAT1, the first response transcription factor in the IFNγ signaling pathway[38]. In our prior research, we had observed that IFNγ stimulation positively influences the expression of the STAT1 gene[27]. This observation led us to consider whether memory might be regulated by controlling the transcription of the STAT1 gene itself. To test this hypothesis, we knocked out the STAT1 gene and next reinserted it into the genome under a constitutive promoter. With such an engineered cell line, we determined that transcriptional regulation of STAT1 has no effect on IFNγ transcriptional memory.

To additionally explore the effect of priming on STAT1, we engineered a cell line by tagging the STAT1 gene with a GFP cassette in a translational fusion. We then used this tool to measure the speed of import of STAT1 to the nucleus in naïve and primed cells, after IFNγ induction. We discovered that STAT1 import is not changed in the primed cells regarding the naïve state.

Our investigation then turned towards understanding the role of STAT1 phosphorylation in the context of transcriptional memory. To do this, we focused on a specific aspect – the phosphorylation of tyrosine 701 (Y701) on STAT1[38]. This modification is a critical step in activating the transcription factor. To explore the role of tyrosine 701 phosphorylation in memory maintenance, we used a small molecule drug. This compound was designed to inhibit a specific enzyme called JAK kinase, which is responsible for phosphorylating STAT1 at the specified position. We administered this drug within the memory window – the period following induction. We found that preventing the phosphorylation of STAT1 during the memory window did not have a significant impact on memory maintenance.

In addition to the Y701 phosphorylation site, there is another site called Serine 727 (S727) known to be phosphorylated on STAT1. This modification is necessary for the full transcriptional activity of the transcription factor[42]. Surprisingly, we found that STAT1 727phosphorylation is maintained long term after removal of IFNγ. This was unexpected, as typical phosphorylation events tend to dissipate after the elimination of the stimulus. To test if this modification of STAT1 plays a role in maintenance of IFNγ transcriptional memory, we engineered a cell line with the STAT1 gene tagged with a degron – dTAG[43]. This enabled us to temporarily remove STAT1 upon addition of a specific small molecule. We discovered that depletion of STAT1 after priming does not impact maintenance of transcriptional memory. Suggesting that stable phosphorylation of STAT1 at position 727, or any other stable modification of the transcription factor, is not the key feature responsible for the persistence of memory. Importantly, it is worth noting that while the consistent maintenance of phosphorylation is not required on a specific STAT1 molecule, the modification might still

play a crucial role in scenarios involving the dynamic addition of the phospho-group, or other modifications.

Finally, we used our STAT1 dTAG cell line to determine if STAT1 is required for memory establishment. During the priming phase, we temporarily removed the transcription factor from the cells using the dTAG system. We found that STAT1 is crucial for the initiation of transcriptional memory. This implies that without STAT1, the memory genes do not undergo the necessary activation for transcriptional memory to take place. This, together with the observation that transcription alone is not sufficient, makes STAT1 the key, specific player in establishment of IFNγ transcriptional memory.

In summary, in this work we showed that the general process of transcription is not sufficient to start IFNγ transcriptional memory. We determined that STAT1 and IRF1 play important roles in regulation of GBP memory genes. Further, we characterized the kinetic response of cells on the molecular level to induction and reinduction with IFNγ and showed increased speed of recruitment of the key transcription factors to their targets. We analyzed specific features of STAT1 activation and found that S727 phosphorylation is maintained long after IFNγ washout, but stable maintenance of the mark is not required for memory persistence. Finally, we proved that STAT1 is fundamental for initiation of IFNγ transcriptional memory *(Fig. 3).*

Wojciech Siwek*, Mariluz Gómez-Rodríguez*, Daniel Sobral, Ivan R. Corrêa Jr and Lars E.T. Jansen. (2018) time-ChIP: a method to determine long-term locus-specific nucleosome inheritance. Methods in Molecular Biology. doi.org/10.1007/978-1-4939-8663-7_7

***Equal contribution**

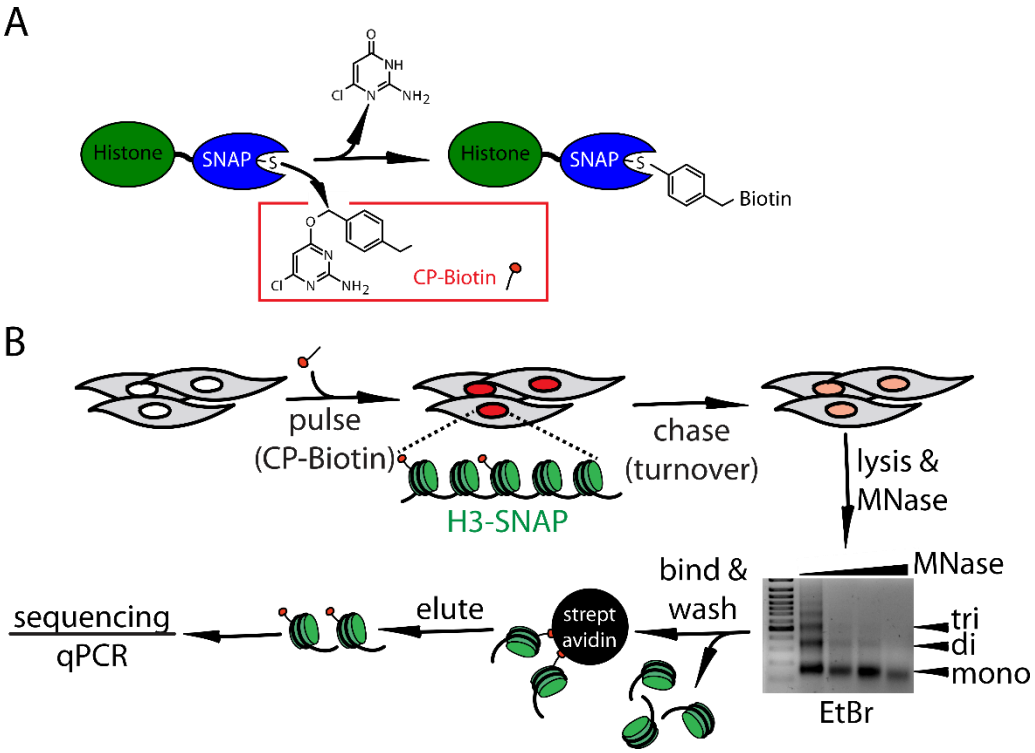

Figure 4. time-ChIP: a method to determine long-term locus-specific nucleosome inheritance. (A) *Reaction scheme of SNAP with CP-Biotin resulting in the covalent self-labeling of SNAP with biotin through a reactive cysteine (S).* ***(B)*** *Outline of pulse-chase time-ChIP assay. Cells expressing SNAP-tagged histone are pulse labeled with CP-Biotin. Following a chase period, the fraction of biotinylated histones retained at nucleosomes decays over time. At specific time points, cells are lysed, nuclei are isolated and chromatin is liberated by MNase treatment. Biotinylated nucleosomes are isolated, purified on streptavidin beads and processed for analysis via qPCR or high throughput sequencing.*

The nucleosome is the fundamental building block of chromatin, which plays a pivotal role in maintaining the expression patterns of genes. This is accomplished through various mechanisms, including the presence of specific histone variants and modifications[44]. To

understand how histones can act as carriers of epigenetic information it is necessary to understand their dynamics in relation to the cell cycle.

There are several methods to measure nucleosome dynamics with positional information (histone kinetics at specific loci). Below, I elaborate on two key techniques.

CATCH-IT (Covalent Attachment of Tags to Capture Histones and Identify Turnover)[45] is based on pulse labeling of endogenous proteins. The nascent proteome is pulse labeled using a derivative of methionine (azidohomoalanine). Subsequently, after a designated chase period a cycloaddition reaction of biotin is performed, followed by chromatin isolation and pull down of labeled nucleosomes. This, combined with genome-wide analysis provides association rates of histones per locus. This method provides valuable insights into how quickly histones are replaced within specific genomic regions. The caveat of this approach is that no specific histone variants can be analyzed. It provides information about histone dynamics in general, but not variant-specific behavior. Further, because this method is based on measuring dynamics of nascent proteins it is not suited for detection of stable pools of nucleosomes.

RITE (Recombination Induced Tag Exchange)[46] provides a way to measure the turnover rates of ancestral groups of specific histones. The core principle of RITE involves using a version of a histone variant that has a constant epitope tag attached. In other words, this tagged histone variant can be easily identified and tracked. Additionally, the gene containing this tagged histone variant is modified so that, when activated by the enzyme Cre recombinase, one epitope tag is swapped out and replaced with another. This exchange is genetic and permanent[46]. One drawback of the RITE system is that it exhibits a delayed response to the induction of Cre recombinase, an updated version of the genes needs to be transcribed, translated and incorporated into chromatin. This means that the tag exchange process might not happen immediately upon induction, introducing a time delay in the experimental process.

Here we aimed to establish a novel method to measure long term nucleosome dynamics genome-wide at high resolution that solves the above-mentioned problems.

The central element of our method is the self-labeling SNAP-tag. SNAP can be pulse labeled in cells, typically by using fluorescent dyes coupled to imaging[47]. Here, we modify this approach by developing a pulse-chase affinity purification strategy, based on a biotin-conjugated SNAP substrate *(Fig. 4A).* Biotin-mediated pulse labeling of SNAP-tagged histones allows us to isolate, and directly measure, histone retention in chromatin at specific loci in human cells. We call this method **time-ChIP** as the pull-down strategy is like a chromatin immunoprecipitation (ChIP) experiment with the added crucial temporal component to determine dynamics of nucleosome occupancy.

SNAP biotinylation can be performed using commercially available BG-Biotin (New England Biolabs). We developed an advanced substrate known as CP-Biotin to achieve more efficient and specific SNAP biotinylation in chromatin in living cells.

To perform chromatin dynamics measurements with time-ChIP, live cells expressing histone-SNAP fusion are pulse labeled with the biotin substrate. Next, the excess label is washed out, cells are chased to allow for histone turnover, nuclei are isolated at specific time points and chromatin is enzymatically fragmented with MNase. Soluble biotinylated chromatin is then isolated on streptavidin and processed for quantitative PCR or high throughput sequencing *(Fig. 4B).*

In a series of proof-of-concept experiments we have combined time-ChIP with qPCR for H3.1-SNAP histone variant and showed that H3.1 can be retained *in cis* on the DNA even during continued transcription and replication. Additionally, we performed time-ChIP coupled to high throughput sequencing for the H3.3-SNAP histone variant, as it presents a known characteristic distribution across the genome linked to gene activity[48]. We next intersected the data with active (H3K9ac, H3K27ac) and inactive (H3K9me3, H3K27me3) parts of the genome and found faster exchange to correlate with active marks, indicating that our H3.3-SNAP fusion protein is behaving as expected. Overall, these results show that the method can be combined with high throughput sequencing to gain insight into locus specific chromatin dynamics.

In summary, in this work we described a novel method, together with a detailed protocol, called time-ChIP. It can measure local histone dynamics and inheritance with both quantitative PCR as well as high throughput sequencing. The method addresses short comings of other similar approaches. It is based on genetically encoded tags, hence allows to perform measurements for specific histone variants. It enables labeling of the whole pull of histone-SNAP protein and so it can be used to measure dynamics long-term. Finally, SNAP labeling is a rapid process which eliminates delays in response to the pulse. The main disadvantage of time-ChIP is low efficiency of biotin labeling (due to limited membrane permeability of the substrate) which requires compensation with relatively high cell numbers. We have addressed this problem, in part, by developing an enhanced biotin label.

4.3.3. Summary of results

To conclude, in this scientific achievement we established a robust system, based on IFNγ stimulation of human cells, to study an important epigenetic phenomenon – transcriptional memory. We characterized the phenomenon in detail on a cellular level and determined that IFNγ transcriptional memory is not a transcription factor feedback loop, hence most likely is controlled via chromatin. Moreover, we discovered that IFNγ transcriptional memory is locally regulated, we showed that the cohesin complex restricts memory establishment *in cis*. On top of that, we determined that the core process of transcription is not sufficient for memory initiation. Further, we pinpointed the key transcription factors taking part in the phenomenon and showed that STAT1 is required for memory establishment. We also discovered a posttranslational modification of STAT1, that persists long after removal of IFNγ.

Aside from work on IFNγ transcriptional memory, we developed a novel method to measure nucleosome dynamics, long term and genome-wide in live cells, called time-ChIP. The procedure can be coupled to both quantitative PCR and high throughput sequencing. It is based on genetic engineering of a specific tag and hence can be used to measure dynamics of specific histone variants. We have confirmed that the method works based on measurements of the H3.3 histone variant and cross section of the results with genomic domains of known histone modifications.

4.3.4. Outlook

Aside from the work presented as part of this scientific achievement, I have been studying IFNγ transcriptional memory by two approaches.

In a hypothesis-based approach, I focused on identification of a chromatin feature that would correlate with the determined length of memory[27]. I focused on the H3K4me as it strongly linked to transcription[49] and was shown (H3K4me2) to be involved in transcriptional memory[24,25,50]. I performed chromatin analysis for naïve, induced and cells 7 days after IFNγ removal. I observed that H3K4me1 (monomethylation) is maintained for at least a week at the memory gene in contrast to H3K4me3 (trimethylation) and me2 (demethylation), which are lost more rapidly (data not published). This is in line with the work from the Natoli lab where the H3K4me1 was shown to correlate with the presence of latent enhancers in macrophages[51].

My observation led to a hypothesis that H3K4me1 cis elements in proximity of memory genes are key for maintenance of IFNγ transcriptional memory.

In an alternative, non-biased approach I performed a genome wide, CRSIPR-Cas9 genetic screen as an independent Marie Skłodowska-Curie research fellow, at the Massachusetts General Hospital, Harvard Medical School. I identified putative factors regulating maintenance but also initiation of IFNγ transcriptional memory.

I am now actively pursuing both of those avenues as a principal investigator at the International Center for Cancer Vaccine Science, University of Gdańsk.

4.3.5. Future plans and potential application

My key aim regarding IFNγ transcriptional memory is to understand how this process works mechanistically on a molecular level. How transcriptional memory is specifically initiated and how it is maintained for long term? I am confident that with the tools, expertise and preliminary data I generated, I will be able to answer the fundamental question of how chromatin-based regulatory mechanisms maintain active transcriptional states.

Finally, I will generalize the knowledge on mechanisms of transcriptional memory to primary monocytes, to pave the way for future biomedical advances. Monocytes show strong trained immunity[52] and can differentiate to macrophages or dendritic cells[53]. These cellular derivatives are present in all tissues[54], are crucial in inflammation and wound healing[55] but also coordinate the adaptive immune response by means of antigen presentation to T cells[56]. My hope is that insights from this research will uncover new avenues for manipulation of the innate immune system for the benefit of health. This will pave the way for industrial collaboration, new jobs in the biotechnology sector and novel immunotherapies.

Obtaining the habilitation degree will enable me to hire PhD students and start working on fulfilling those goals.

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5. Presentation of significant scientific activity carried out at more than one scientific institution

From the early steps of my scientific career, I have been striving for independence. During my MSc studies at the University of Warsaw, PL I had the privilege to work in a laboratory that enabled me to pursue my own research project. During this time, I became fascinated with molecular mechanisms of gene regulation and have been following this path ever since. Moreover, I completed an international internship at a world-renowned research institute, the Sainsbury Laboratory at the John Innes Center, UK.

To gain deeper understanding of gene regulation, during my PhD studies at the International Institute of Molecular and Cell Biology, PL I focused my efforts on structural biology of protein-DNA interactions. I took the initiative to design my own research project, successfully convinced my PhD advisor to support this direction, secured a scholarship (from the Mazovian Voivodeship) and research funding, as a principal investigator (from the National Science Center, NCN). This resulted in the discovery of a structural mechanism underlying specific recognition of methylated DNA by proteins. The research gave rise to two high impact publications (among others) in an international peer-reviewed journal on which I am the first and for the second one also the corresponding author (*Siwek et al. Nucleic Acids Research, 2012 & Mierzejeska, Siwek et al. Nucleic Acids Research, 2014*). Within the time of my PhD project, I kick started a productive and long-standing collaboration with a mass spectrometry laboratory and the results of that partnership became part of the published work (*Mierzejeska, Siwek et al. Nucleic Acids Research, 2014*), as well as my thesis. I wrote the manuscripts resulting from my work and mentored my younger colleagues. Moreover, in that time, I completed a postgraduate study in Methods of Analytical Chemistry at the University of Warsaw, PL.

For my postdoctoral training, initially at Instituto Gulbenkian de Ciência, PT and later at the Department of Biochemistry, University of Oxford, UK, both with Lars Jansen, I decided to expand and consolidate my interests in gene regulation above structural biology to a more physiological model system. In the laboratory, I spearheaded a novel theme centered on gene regulation in mammalian cells that went above and beyond the host laboratory established expertise. I discovered a mechanism by which transcriptional memory / trained immunity is established in human cells (*Siwek et al. Molecular Cell, 2020*). On that work, I am the first and corresponding author. I was also a co-advisor to a PhD student. The collaboration resulted in a publication, on which I am a corresponding author (*Tehrani et al. EMBO journal, 2023*).

Additionally, I developed a novel method to measure nucleosome dynamics that resulted in a first author publication (*Siwek et al. Methods in Molecular Biology, 2018*). For my postdoctoral training, I secured a fellowship and as a co-PI with my mentor, a research grant from the Portuguese state funding agency.

Achievements from my postdoc, as well as the tools and assays I developed during that time, allowed me to secure a Marie Skłodowska-Curie global fellowship to continue my work, on transcriptional memory, as an independent research fellow at the Massachusetts General Hospital (MGH), Harvard Medical School, USA in partnership with the International Centre for Cancer Vaccine Science (ICCVS) at the University of Gdańsk, PL. More recently, I secured an assistant professor position at the ICCVS, together with a start-up package and obtained a well-funded Sonata grant from the National Science Center, NCN to study the molecular mechanisms of IFNγ transcriptional memory.

In summary, my scientific journey has been marked by a consistent drive for independence, impactful research, and collaborative leadership. From the earliest stages of my career to my current roles as an assistant professor and independent researcher, I've taken initiatives, designed projects, secured funding, mentored colleagues, and contributed significantly to our understanding of gene regulation and transcriptional memory.

6. Presentation of teaching and organizational achievements as well as achievements in popularization of science

I firmly hold the belief that scientists bear a responsibility to actively engage with society beyond their research pursuits. This commitment has guided my efforts to contribute meaningfully to both scientific and public spheres.

During my MSc studies, I took my first proactive step towards fulfilling this commitment. Together with the Centre for Innovative Bioscience Education in Poland (BioCen), I co-organized a laboratory training workshop tailored for high school students. This initiative aimed to provide these young minds with a firsthand experience of a laboratory environment, fostering curiosity and enthusiasm for bioscience.

During my PhD studies, I spearheaded a scientific discussion club “DoScience”. Within the initiative, I co-organized more than 25 scientific meetings, open to the public. Notably, two major events sponsored by EMBO featured Nobel laureates Venki Ramakrishanan and Brian Kobilka. In addition to this, I embraced mentorship roles, co-advising an MSc student named Marta Doliwa and providing guidance to an undergraduate student.

During my postdoctoral work, I was a member of the postdoc committee at the Instituto Gulbenkian de Ciência, PT. Amid on the committee, I co-organized a peer review workshop sponsored by the journal eLife. Beyond this, I was a lecturer at the Instituto Gulbenkian de Ciência, PT, PhD Program – structural and molecular biology module. Moreover, I co-advised a PhD student named Sahar Tehrani and guided an undergraduate student. My contributions extended to being a part of the scientific committee for the International Young Scientists Conference on Molecular and Cell Biology in 2020.

I am also an ad hoc reviewer for several scientific journals including Epigenetics and Cancer Communications.

7. Other important information about professional career

Throughout my scientific journey, I actively pursue hands-on training to enhance my skill set. From 2015 to 2018, I participated in the Gulbenkian Training Program in Bioinformatics. This comprehensive program spanned six intensive courses, providing a thorough exploration of various aspects of bioinformatic data analysis. Additionally, during my time in the United States, I undertook a nine-month course on Leadership in Research, conducted by Harvard University. This intensive training was a pivotal step in refining my abilities in laboratory management and equipping me with essential tools for establishing and leading a research group at the University of Gdańsk.

I declare that I have not previously applied for the degree of habilitation.

Signed by /
Podpisano przez:

Wojciech
Mieczysław Siwek
Uniwersytet Gdański

Date / Data:
2023-09-25 11:31

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(Applicant's signature)