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Review of a PhD thesis

entitled "Furthering the understanding of mechanism and function of HCMV IE1 chromatin association"

by MSc Mamata Sumant Savanagouder

The aim of Ms Mamata Sumant Savanagouder thesis is to contribute to the understanding of the complex mechanism of human cytomegalovirus (HCMV) control of latency and lytic cycle. HCMV is the largest human herpesvirus, with a large genome encoding almost 200 proteins as well as numerous non-coding RNAs. Moreover, the variability of the HCMV sequence, the changeable mode of gene expression and the diversity of interaction with host cell makes this virus, despite its ubiquitous presence in human populations, a puzzle and a challenge for researchers. Therefore, the topic of Ms Savanagouder PhD project is well chosen and the data presented in her thesis add valuable information to HCMV molecular studies.

Unlike alpha-herpesviruses, which establish latency in neuronal non-dividing cells and unlike gamma-herpesviruses which latency occurs predominantly in quickly proliferating B cells , HCMV , a betaherpesvirus, can persist in a latent form in many kinds of cells. The mechanism of latency control is therefore very complex and may differ depending on the type of host cell. This diversity led to many questions about HCMV true molecular latency or rather a low-level persistence of productive infection. There are still many gaps in our knowledge of mechanism of HCMV latency. The focus of Ms Savanagouder project was the investigation of the role of the immediate-early 1 (IE1) protein in latency establishment in various types of cells. The

of IE1 in the mechanism of association of viral genome with host chromosomes was examined in the view of its potential functional similarity to maintenance proteins (MPs) of Epstein -Barr virus (EBV) EBNA protein and Kaposi Sarcoma (KHSV) virus LANA protein. The expertise of the promotor of Ms Savanagouder thesis, dr hab. Magdalena Weidner- Glunde, who studied for long time the latency mechanisms of gamma-herpesviruses , provided very sound support for this PhD project.

Among the several IE proteins involved in control of HCMV gene expression , Ms Savanagouder focused her attention on the small protein IE1X4 , the product of exon 4 in MIE (major immediate early) region. This protein has been reported by other authors as a candidate for HCMV latency-specific factor that promotes viral chromosome maintenance and replication. The investigation of IE1x4 is the main topic of the first part of Ms Savanagouder PhD research.

She started her work with cloning IE1x4 gene and checking its expression in transfected cells, carefully testing the influence of various positions of fused tags on protein properties. To examine the function of this protein, she studied its localization in two types of cells using confocal microscopy. The confocal imaging is the essential experimental method used throughout the whole PhD thesis, therefore the Author evidently put a lot of effort to obtain good quality images. This technique was applied to visualize the differences between full and short form of IE1 localization and to validate the hypothesis of possible role of IE1x4 as maintenance protein (similar to LANA or EBNA) which could be involved in tethering HCMV genome to cellular chromatin.

The experiments did not confirm this hypothesis although some new observations about the IE1 "painting" or spots pattern on chromosomes have been made. As the Author concluded in discussion, these results might be host specific, different in other types of cells ; the conditions of the in vitro experiments could also affect the results. As the next step, the Author used T98G cells to establish a model of latency (here it would be good to explain the abbreviation and characterize these cells) which has been validated by checking the expression of IE and late genes in a long time period (up to 35 days) in infected cells. The decreasing level of expression of individual genes and loss of viral progeny production indicated the transition to latency. The production of IE1x4 protein was detected neither in this model nor in another cell line (Kasumi-3) what further indicated that this protein does not directly play the role of maintenance protein during latency. However , the presence of IE1 transcript even after the start of latency in infected model cells suggested that this region may be involved in an unknown way in latency control. In further studies presented in the thesis , Author followed her observations of the presence of IE1 in chromosome associated spots (CAS) in a panel of several types of cells of various origin. Analysis of this novel localization was carefully validated by

checking all the protocols , various fixation techniques etc. The interesting information obtained from this part of the study was the detection of IE1 CAS only in tumor cell lines which facilitated HCMV latency establishment. Moreover, the double spots were observed only near primary constriction sites and only in part of the chromosomes. To analyze in more detailed way the IE1 chromosome-associated spots localization pattern and the association of IE1 with chromatin , Ms Savanagouder generated a number of IE1 mutants, including mutants with deleted functional regions, with point mutations and clustered mutations. In this part, by cloning individual domains of IE1, she managed to determine that a long core domain, located outside of IE1 chromatin tethering domain (CTD), is important for IE1 CAS localization pattern, however no individual residues in this domain were identified as essential for CAS formation. The other observation made in this part of the thesis is the impact of level of IE1 protein expression on the frequency of IE1 CAS formation . The experiments performed on transfected cells showed that (to a certain threshold) increased level of IE1 resulted in higher number of cells with CAS localization pattern. These experiments were followed by the monitoring IE1 CAS formation in infected cells, which led to the conclusion that the establishment of latency is accompanied by the increase of number of cells with spots. Such observation suggests that changes in IE1 localization may reflect the switch between lytic and latent phase of HCMV life cycle, which could shed new light on biological function of IE1 protein.

As the large number of experiments described in Ms Savanagouder have been performed in cells of neural origin , glioblastoma T98G cells , the collection of IE1 observations made in this project may be significant for the studies of the role of HCMV in the development of brain tumors. The oncomodulatory potential of HCMV is highly studied and highly controversial – like most research problems in the complicated biology of this virus. However, there is no doubt about persistence of the virus in the host organism and about complex mechanisms of its dynamic interaction with various types of cells, including tumor cells. Therefore, it is important that Ms Savanagouder used a large variety of cells in her studies , comparing her observations i.e. about IE1 localization in the context of different cells.

Generally , the experimental work presented in Ms Savanagouder is thematically coherent and related to important aspects of HCMV biology. All experiments were logically planned and set with proper or even scrupulous controls. The very high quality of images facilitates the interpretation of the results (based in large extent on confocal microscopy). The important aspect of this project was the comparison of mechanism of latency control in various herpesviruses , not only by literature data but also to some extent experimentally, as the localization of IE1 spots was compared with the images of spots formed by KHSV LANA protein in the same cell line. The final conclusion from these series of experiments is that HCMV

ensures persistent maintenance of its genome during cell division in a way more similar to the strategy used by human papilloma virus (HPV 8) than the one used by gammaherpesviruses.

Here my question is : what could be another way, apart from colocalization studies, to investigate the mechanism of tethering of viral genome to host chromosomes ? Can the interaction between potential maintenance proteins and specific regions of the host chromosomes be proven in some biochemical way?

The experimental part is preceded by an abstract and the theoretical introduction. The introduction gradually leads the reader from the general properties of herpesviruses and human cytomegalovirus to the characterization of latency, immediate-early proteins and maintenance proteins. The mechanism of association of CMV and other viruses' genomes with host chromosomes is presented in a more detailed way. The important aspect of HCMV oncogenic potential with the focus on glioblastoma is presented in the last part of the introduction, preceding the description of the thesis aims. The theoretical part is well written and contains the essential background information , helping to understand the aims of the project. However , for any reader who (like most molecular biologists) uses on the everyday basis the HCMV promoter in expression plasmids, the transactivator function of MIE region and the structure of the promoter, as the main regulator of HCMV infection , both during lytic cycle and reactivation from latency, could be described in a bit more detailed way. Some introduction about the significance and interpretation of the appearance of the spots ("speckles") versus "painting" pattern in the analysis of protein function (also with regard to PML an CENP-A localization) would also be helpful. The aims of the project are formulated in an unusual way, more like an abstract, describing the results of consecutive steps of the project , already with some conclusions, but the ideas and reasons for subsequent research steps can be found in this chapter.

The materials and methods part contains the comprehensive description of the technical part of the project and only be reading this chapter I have realized all the experimental effort of Ms Savanagouder project. Apart from many basic molecular biology methods she also used advanced cell biology technologies, like the generation of induced pluripotent stem cells (iPSC) from human placenta fibroblasts and differentiation of iPSC to neural stem cells, needed for her studies. I think that part of the work should be also included in the results with some explanation why it was important to obtain these cells. The other challenging experimentally task was the construction of HCMV mutant. That part is also not described in the "results" chapter. My question here is: what was the purpose of the construction and how this mutant was characterized?

The huge number and quality of confocal images presented in the thesis shows excellent microscopy skills of the Author. For any researcher familiar with such visualization techniques , it is obvious that they were

obtained by many hundreds of hours spent at the microscope, what, together with the length of time required by some experiments , testifies about heavy workload put in this project. The experimental part is well illustrated, mostly by images obtained from transfected or infected cells and by the results of Western blotting analysis.

All the results are thoroughly discussed in the last chapter of the thesis. In the well-written discussion the Author describes the most important results of her work, comparing them with many reports from other laboratories and discussing the possible reasons for differences. She indicates the areas where further investigation is needed to answer the question how HCMV genome tethering to chromatin is mediated and what is the role of IE1 in this mechanism. The analysis presented in this chapter is very thorough and the author presents convincingly her arguments about the possible role of CAS formation during latency establishment, similarity of IE1 to HPV protein E2 as well as her own hypotheses about oncomodulatory impact of IE1 expression in tumor cells. The discussion is substantiated by presenting various interpretation of data and solid knowledge of literature , accumulated from 259 references. The discussion ends with a short summary of main conclusions. However, considering a large volume of various data included in the thesis, a separate list of all conclusions drown from this project would be helpful for the reader.

The long list of abbreviations is a useful part of the thesis (some abbreviations are still missing) and very personal "acknowledgements" chapter helps to understand not only how important is the scientific support for a PhD student but also how challenging is the life of a researcher in a foreign country.

Summarizing , the project brings new insight into the role of IE1 protein in latency and persistence of HCMV . The data presented in the thesis well reflect the complexity of molecular biology of this virus.

I conclude that the presented dissertation fulfills all the requirements for a PhD thesis specified in Article 187 (1-4) of the Law on Higher Education and Science (Journal of Laws 2018, item 1668, as amended). Therefore, I am applying to the Council of the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences for admission of Ms Mamata Sumant Savanagouder to further stages of the doctoral procedure and I support the application to grant her the doctor of philosophy (PhD) degree.

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