**To the ISF review committee**

**Re: Re-submission rebuttal letter - application- #547/22**

We would like to thank the ISF committee and the reviewers for their constructive comments. These were carefully read and implemented in the revised application. Based on the reviewers’ requests, new preliminary data that are described below were added to the proposal. The following letter summarizes the new preliminary results that were included in our study, followed by a rebuttal letter for each of the reviewers’ comments.

**The following preliminary data has been added to the revised application**:

1. **Studying HIV latency in resting CD4 primary T cells** **–** In our revised study, we addressed the issue of the cell model that was used for our preliminary results. We clarified that the use of a Jurkat-derived HIV-infected T cell line (J-Lat 6.3) was mainly due to its amenability to Cytor gene manipulation and efficient HIV infection. Moreover, our screen for lncRNAs in the J-Lat 6.3 cells only documented changes in the transcriptome in response to T cell stimulation, allowing us to identify coding genes and ncRNA that exhibit a significant shift in their expression following such stimulation. However, while we continue to rely on the J-Lat 6.3 cells for proof-of-principle studies and gain and loss-of-function experiments for studying lncRNAs as regulators of HIV gene expression and latency, we have extended our study and incorporated additional new preliminary results regarding the effects of Cytor on HIV infection in primary CD4 T cells. First, the expression of lncRNAs exhibiting the strongest expression shift in J-Lat 6.3 cells was confirmed in primary CD4 T cells under resting and stimulated conditions (**Fig. 1D; p3**). Additional preliminary results obtained in primary CD4 T cells are described below in the context of their relevant point that was raised by the reviewers. Furthermore, a difficulty in studying HIV latency in resting CD4 T cells is that they are refractory to HIV infection. To overcome this limitation, we have established the gold standard model for studying HIV latency whereon isolated primary CD4 T cells T are first activated and then subjected to the manipulation of Cytor expression by shRNA and HIV infection. Cells are cultured without stimulation until latency is established and then reactivated and scored for latency reversal in the context of Cytor gene manipulation (**SA1; p10**). Since such post-activated CD4+ primary T cells are not truly resting cells, the above-described protocol will impact HIV gene expression and latency reversal. Therefore, we will use a CRISPR-mediated methodology for manipulating Cytor levels in truly resting CD4 T cells without activating these T cells (**see** **Fig. 6;** **p7**). **SA1** of the revised proposal plans to optimize this protocol by employing CRISPRi/a to manipulate Cytor levels and score for Cytor effects on latency establishment and reversal in resting primary T cells. Furthermore, the revised proposal will extend our observations regarding the effects of Cytor and confirm its functional relevance in the context of HIV infection, latency establishment, and reversal in primary resting CD4 T cells.

**New preliminary results that are presented in the revised study:**

1. Levels of identified lncRNAs were documented in primary CD4 T cells, showing a significant shift in their RNA levels when comparing resting or stimulated conditions (**Fig. 1D; p 3**(.
2. In activated CD4+ T primary cells, depletion of Cytor by shRNA inhibits HIV gene expression and promotes latency (**Fig. 8; p8**).
3. Upon the stimulation of primary CD4 T cell, Cytor translocates to the nucleus and its levels are elevated in the nucleus relative to 7SK ncRNA, which is known to interact with inactive P-TEFb (**Fig. 7; p7**).
4. **Cytor effects on HIV gene activation are indirectly linked to its ability to control T cell activation and actin remodeling** **–** Our RNA-Seq analysis demonstrated that Cytor knockdown led to a broad effect on cellular gene expression. GO analysis further revealed pathways that are involved in actin remodeling, T cell activation, and gene transcription (**Fig. 5; p6**). Based on these results, **we hypothesize that the effects of Cytor on HIV gene expression may be indirectly coupled to T cell activation and actin dynamics through the regulation of Cytor target genes*.*** Since Cytor was previously shown to regulate cellular actin dynamics and cytoskeleton reorganization in fibroblasts, but not in CD4 T cells, and its effects on HIV are not known, **we present preliminary results demonstrating that in Jurkat T cells, Cytor plays a role in T cell activation and cellular actin dynamics. Depletion of Cytor expression reduced the number of cells that responded to T cell activation with proper spreading and F-actin polymerization** (**Fig. 4; p6**). Our revised proposal will extend these preliminary data into primary CD4 T cells (**SA3**). We will also confirm the RNA-Seq analysis in primary CD4 T cells, where Cytor levels will be depleted under active or resting cell states. New Cytor target genes will be identified followed by gain- and loss-of-function experiments designed to elucidate the role of these Cytor target genes in T cell activation and actin remodeling. These studies will be performed in the context of Cytor expression manipulation to understand the role of Cytor in the functions of these target genes. Finally, other mechanistic pathways that may be relevant to the indirect effects of Cytor on HIV gene expression will be explored. These include effects through miRNAs that bind Cytor, or via transcription factors that associate with Cytor and activate HIV transcription such as Wnt/b-Catenin, NFkB, and JAK/STAT.
5. **Studying direct molecular mechanisms by which Cytor activates HIV gene expression**: As rightly requested by the reviewers, **we have modified our proposal and added new preliminary results**.
6. We confirmed interactions between P-TEFb and endogenous Cytor by RNA pull-down combined with Mass Spectrometry using an antisense Cytor probe that targets the endogenous Cytor within cells. We further showed that P-TEFb/SEC associates with Cytor through a RIP analysis performed using similar oligos (**Fig. 3D; p5**). **SA2** of our revised work will extend these preliminary results and define the direct effects of Cytor on HIV gene expression. We will establish direct interactions between Cytor and P-TEFb both in cells and *in vitro*. Accordingly, we modified the study to accurately describe the methodology that will confirm the direct association between endogenous Cytor and P-TEFb/Cyclin T1 in cells. These approaches will include RNA pull-down with anti-sense biotinylated oligos that target endogenous Cytor, followed by western blotting to detect P-TEFb and RT-qPCR to confirm Cytor levels. Experiments will be conducted initially in T cell lines and then in primary HIV-infected CD4 T cells under resting or stimulated conditions. ChIRP-qPCR will be further employed, using antisense oligos to confirm Cytor occupancy on the HIV promoter in primary CD4 T cells under resting or stimulated conditions.
7. New preliminary results in primary CD4 T cells describe the dynamics of Cytor localization between cytoplasmic and nuclear fractions under resting or stimulated conditions. In addition, relative Cytor levels in the nucleus and its association with P-TEFb are increased upon cell stimulation (**Fig. 7; p7**).
8. **The interplay of Cytor with Tat and 7SK lncRNA:** As correctly pointed out by the reviewers, the interplay between Cytor and 7SK ncRNA is an interesting aspect of the study (**see Sub-Aim 2d; p13**). We will therefore explore the interplay between Cytor, HIV-Tat, and 7SK lncRNA in the context of HIV gene activation and the regulation of P-TEFb activity. The new experimental design will include EMSA analyses that will test the recruitment of Cytor by Tat. Furthermore, Cytor effects on HIV transcription will be tested with a virus that does not express Tat or a mutated Tat that does not bind cyclin T1. Additionally, Tat trans-activation will be monitored in Jurkat cells under conditions where Cytor expression is modulated. Tat association with Cytor will be monitored by RNA-IP, followed by RT-qPCR. We will also extend our preliminary findings regarding the interplay between Cytor and 7SK ncRNAs in primary CD4 T cells. Upon cell stimulation, the association of Cytor with P-TEFb is increased relative to that with 7SK lncRNA, and it also translocates into the nucleus (**see** **Fig 7; p7b**). Additional glycerol gradient analysis and western blotting will be conducted to identify proteins that are present in Cytor or 7SK lncRNA complexes together with P-TEFb. RT-qPCR will reveal the association of Cytor and 7SK RNAs in free versus large complexes.

**Specific answers for reviewer comments**:

**Reviewer #1**

We thank the reviewer for their valuable comments. As suggested, in the revised research plan we have thoroughly addressed the point raised by the reviewer regarding the use of the Jurkat-derived HIV-infected J-Lat 6.3 cell model for studying HIV latency. As summarized above (**point #1**), usage of these cells was mainly due to their amenability to the manipulation of Cytor expression and efficient HIV infection. Our revised study presents new preliminary data in CD4 primary T cells, showing that: (**1**) cell stimulation leads to a significant shift in levels of identified lncRNAs, including Cytor (**Fig 1D. p 3**); (**2**) Following activation of CD4 primary cells, levels of Cytor RNA in the nucleus are elevated; (**3**) the association of Cytor with P-TEFb is elevated relative to that with 7SK ncRNA, which is known to associate with P-TEFb and inactivate its kinase activity (**Fig. 7**; **p7**); (**4**) Cytor depletion inhibits HIV gene expression in stimulated primary CD4 T cells (**Fig. 8; p8**). Moreover, as these primary cells are not truly resting cells that constitute the HIV latent reservoir, we have established in our lab protocols for Cytor gene editing in truly resting CD4 primary T cells using CRISPR tools (**Fig. 6; p7)**.

**Reviewer #2**

This reviewer correctly raised concerns about the relevance of the J-Lat 6.3 cells for studying HIV latency. In the revised proposal, we indeed state that the screen in J-Lat 6.3 cells identified ncRNAs only based on their altered expression levels following T-cell stimulation. Therefore, as pointed out, gain- and loss-of-function experiments will further validate the potential roles of these ncRNAs as regulators of HIV gene expression and latency. These assays will be performed in Jurkat-derived cells and primary CD4 T cells under resting and stimulated conditions. Preliminary data presented in **Fig. 1D; p3**, show that levels of ncRNAs, including Cytor, were significantly shifted upon the stimulation of primary CD4 T cells. Although we will continue to rely on the Jurkat-derived cells for biochemical and functional analysis, our revised work will combine experiments that will test Cytor effects in primary resting CD4 primary cells that primarily constitute the HIV reservoir. Accordingly, methodologies for working with these cells and manipulating Cytor levels have been established in our lab (**Fig. 6-8; see above point #1**). In addition, new preliminary results show that upon depletion of Cytor in stimulated primary CD4 T cells, HIV infection is inhibited (**Fig. 8; p8**); Moreover, Cytor levels are elevated in the nucleus following CD4 primary T cell stimulation, and its association with P-TEFb in the nucleus is increased upon cell stimulation (**Fig 7; p7**).

**Additional specific points raised by the reviewer:**

1. We have modified the overall goal of the revised study. Accordingly, following our screen, we will validate the functional relevance of identified lncRNAs for HIV infection by employing gain- and loss-of-function experiments in T cell lines and in resting primary CD4+ T cells that are the physiologic cell targets of HIV.
2. “**Cytor effects on HIV gene expression and latency may be indirect via regulation of Cytor target genes” –** The revised **SA3** will explore the indirect effects of Cytor on T cell activation, actin remodeling, and cellular or HIV gene expression. RNA-Seq analysis identified specific Cytor gene targets that are differentially expressed upon Cytor depletion (**Fig. 5; p6**). GO analyses revealed these targets to be enriched in the gene transcription, T cell activation, and actin remodeling pathways. New preliminary results in Jurkat-derived CD4 T cell lines show that Cytor depletion disrupts proper T cell activation and actin dynamics **(Fig. 4; p6).** Based on these preliminary findings, **we hypothesize that in primary CD4 T cells the effects of Cytor on T cell actin dynamics may be mediated by the regulation of Cytor target genes, thereby indirectly affecting HIV gene expression and viral latency.**We plan to identify these target genes of Cytor and investigate their role in T cell activation, actin dynamics, and HIV gene expression in T cell lines and primary cells. Finally, other mechanistic functions of Cytor target genes that indirectly regulate HIV gene expression cannot be excluded and will be also explored.
3. As raised by the reviewer, the interplay between Cytor, 7SK lncRNA, and Tat will be studied in the context of HIV infection - **above point #4; page 13 in the revised proposal.**
4. As suggested by the reviewer, an accurate description of the methods for analyzing RNA-protein interactions is described in the revised proposal – **please refer to SA2 in the revised study and point #3 above**.We have also repeated the RNA-pull down assay followed by MS in J-Lat 6.3 cells using an antisense oligo targetting endogenous Cytor. We thereby identified Cytor protein partners including P-TEFb and SEC. These MS results were validated by RIP-WB and included the appropriate controls as requested (**Fig. 3D; p5**)**.**
5. We have added new preliminary RT-qPCR results performed in combination with cell fractionation and show that levels of Cytor in the nucleus are elevated following the stimulation of primary CD4 T cells. We further show that Cytor association with P-TEFb is increased upon cell stimulation (**Fig.7; p7).**
6. Methods for documenting Cytor-Cyclin T1 interactions – we have accurately revised the description of the methods that describe the interactions between Cyclin T1 and endogenous Cytor and their recruitment to the HIV promoter (**SA2; p12**). *In vitro* RNA-protein binding assays will complement these experiments and confirm direct Cytor binding to cyclin T1 (**2b; p12**).
7. As suggested, we have obtained the HIVGKO clone from the Verdin lab and now show infection experiments in primary CD4 T cells (**Fig. 8; p8**).

**Reviewer #3**

We have addressed the reviewer’s concerns regarding the use of the J-Lat 6.3 cells in the screen for ncRNAs (**also see point #1)**. We will combine work studies of these cells with experiments in primary CD4 T cells to confirm a change in ncRNA levels. New preliminary results in primary CD4 T cells demonstrate that upon stimulation, HIV infection is inhibited in cells where Cytor is knocked down (**Fig 8; p8**). Additionally, stimulation of primary CD4 T cells leads to the translocation of Cytor to the nucleus, and the levels of Cytor that are associated with P-TEFb are elevated (**Fig 7; p7**). **SA1** in the revised work will exploit primary resting CD4 T cells to score for latency establishment and reversal in the context of Cytor manipulation. Protocols that will be used to manipulate Cytor expression in resting CD4 primary T cells via CRISPRi/a have been established (**Fig. 6; p7**). We have also repeated the gain- or loss-of-function experiments for Cytor in J-Lat 6.3 T cells and found that depletion of Cytor led to a decrease in HIV-GFP expression (**Fig. 3B; p4**). As requested, controls to monitor the association of Cytor with P-TEFb in the RIP assay were included in our revised study and included a scramble RNA probe and an antisense 7SK lncRNA oligo (**Fig. 3D; p5**). As requested for the ChiRP-qPCR assay, a control of an unrelated gene locus was added (**Fig. 3A; p5**). In **point #2** above, we present new data regarding the indirect effects of Cytor on T cell activation, actin remodeling, and subsequently HIV gene expression. **SA3** will identify Cytor-gene targets and study their role in T cell activation, actin remodeling, and HIV gene expression in CD4 primary cells (**p14**).

**Specific points raised by the reviewer:**

1. As suggested, in our revised work we have outlined alternative cell lines that will be used to avoid biases originating from changes in proviral integration sites (**p14**).
2. We have revised the methods used to monitor binding between endogenous Cytor and P-TEFb by ChIRP. Antisense oligos will target endogenous Cytor.
3. Gain- and loss-of-function studies confirmed a 4-fold decrease in HIV gene expression upon Cytor depletion (**Fig 2b; p4 in the revised proposal**).
4. **Fig. 10** **p13** –the ChIP-seq ENCODE data of P-TEFb global occupancy show Cytor target genes that were downregulated upon Cytor KD as shown in the RNA-Seq analysis.
5. In revised RNA pull-down and ChiRP assays (**Fig. 3; pp5**), appropriate controls of irrelevant RNA (7SK lncRNA), a scrambled probe, and downstream gene locus were included. We will conduct pull-down assays to monitor P-TEFb association with endogenous Cytor using antisense probes.
6. We focused our study on the indirect effects of Cytor, primarily on T cell activation and actin dynamics. **SA3** will identify new Cytor gene targets. Their expression will be manipulated in the context of Cytor gene manipulation.

**Reviewer #4**

As correctly raised by this reviewer, the interplay between Cytor and 7SK lncRNA and Tat will be investigated in the revised work (**point #4 above**).

**Reviewer #5**

This reviewer expressed concerns regarding the J-Lat 6.3 model. As stated **in point #1**, we will confirm our study and results in resting CD4 primary cells and score productive viral infection in these cells. We have repeated the ChIP- qPCR analysis, as requested (**Fig. 3B-C; p5**).