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NOTES

Role of Protein Kinase C δ in Reactivation of Kaposi's Sarcoma-Associated Herpesvirus

Einat Deutsch, Adina Cohen, Gila Kazimirsky, Sara Dovrat, Hadara Rubinfeld, Chaya Brodie, and Ronit Sarid*

Faculty of Life Sciences, Bar-Ilan University, Ramat Gan, Israel

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TPA (12-*O*-tetradecanoylphorbol-13-acetate), a well-known activator of protein kinase C (PKC), can experimentally induce reactivation of Kaposi's sarcoma-associated herpesvirus (KSHV) in certain latently infected cells. We selectively blocked the activity of PKC isoforms by using GF 109203X or rottlerin and demonstrated that this inhibition largely decreased lytic KSHV reactivation by TPA. Translocation of the PKCδ isoform was evident shortly after TPA stimulation. Overexpression of the dominant-negative PKCδ mutant supported an essential role for the PKCδ isoform in virus reactivation, yet overexpression of PKCδ alone was not sufficient to induce lytic reactivation of KSHV, suggesting that additional signaling molecules participate in this pathway.

Kaposi's sarcoma (KS)-associated herpesvirus (KSHV), also known as human herpesvirus 8, is causally implicated in KS, primary effusion lymphoma (PEL; also known as body cavity-based lymphoma), and a subset of multicentric Castleman's disease (1, 10, 47, 48). Like all other herpesviruses, primary infection with KSHV precedes lifelong latent infection, while virus reactivation may occur and lead to an increased risk for disease development (21). Only a few viral proteins are expressed during KSHV latency, whereas extensive KSHV genome expression and productive viral DNA replication characterize the lytic phase of virus infection (19, 29, 43, 46). Detection of KSHV in peripheral blood mononuclear cells and KSHV seropositivity are strongly predictive of the development of KS, whereas active replication of KSHV in circulating lymphoid cells is likely responsible for the spread of virus to the endothelium and the onset of KS (8, 51, 62). Relatively little is presently known about the host and cellular factors that can affect and play a role in the intracellular signaling pathways of virus reactivation.

Major tools for studying KSHV biology are latently infected B-cell lines, derived from patients with PEL, in which the virus undergoes spontaneous lytic reactivation in a small steady fraction of the cells (44, 46). Increased, but limited, virus reactivation is observed following exposure of these cell lines to a variety of stimuli such as interleukin-6 (IL-6) (9, 11, 52) and gamma interferon (9), hypoxic conditions (16), coinfection by another viral agent (27, 36, 57), and treatment with chemical reagents such as *n*-butyrate (37), ionomycin (9, 67), 5-azacytidine (12), and the potent protein kinase C (PKC) activator 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (39, 44). In addition, ectopic expression of the KSHV lytic replication and transcription activator (KSHV/Rta), encoded by viral open reading frame (ORF) 50, is generally sufficient to disrupt virus latency and induce lytic virus reactivation (33, 61). Thus, it is likely that at least part of the effect of agents that activate the virus lytic cycle is through the transcriptional and posttranscriptional activation of this gene; yet, the upstream signaling cascades that influence the expression of KSHV/Rta have not been fully elucidated (7, 12, 22, 26, 32, 33, 41, 61).

The PKC family, comprised of 12 structurally related lipid-regulated serine-threonine kinases, plays a central role in the transduction of a variety of signals that affect cellular functions and proliferation (45). Diacylglycerols (DAG) and calcium ions are the naturally occurring activators of certain members of this family. Phorbol esters, such as TPA, compete with DAG for the same binding site and function as potent PKC agonists (2, 17, 49). Yet, nonkinase DAG and phorbol ester receptors, such as the Ras guanyl releasing protein (RasGRP) and chimaerins, have also been described previously (18, 45, 55).

Our study was designed to determine the role of PKC in KSHV lytic reactivation by TPA and to identify specific PKC isoforms that contribute to the disruption of the latency of KSHV and to virus reactivation. We demonstrate that the activity of PKCδ is required, yet not sufficient, for TPA-mediated virus reactivation.

Selective inhibitors of PKC isoforms inhibit KSHV lytic reactivation. To establish the role of PKC in KSHV lytic reactivation, we investigated the effects of selective PKC inhibitors in PEL-derived KSHV-infected BCP-1 (5) and BCBL-1 (44) cell lines. These experiments were crucial, since not all phorbol ester responses can be attributed to the activities of PKC isoforms (45). As previously reported, we obtained KSHV lytic reactivation after TPA stimulation (39, 44, 46).

* Corresponding author. Mailing address: Faculty of Life Sciences, Bar-Ilan University, Ramat Gan 52900, Israel. Phone: 972-3-5317853. Fax: 972-3-5351824. E-mail: saridr@mail.biu.ac.il.

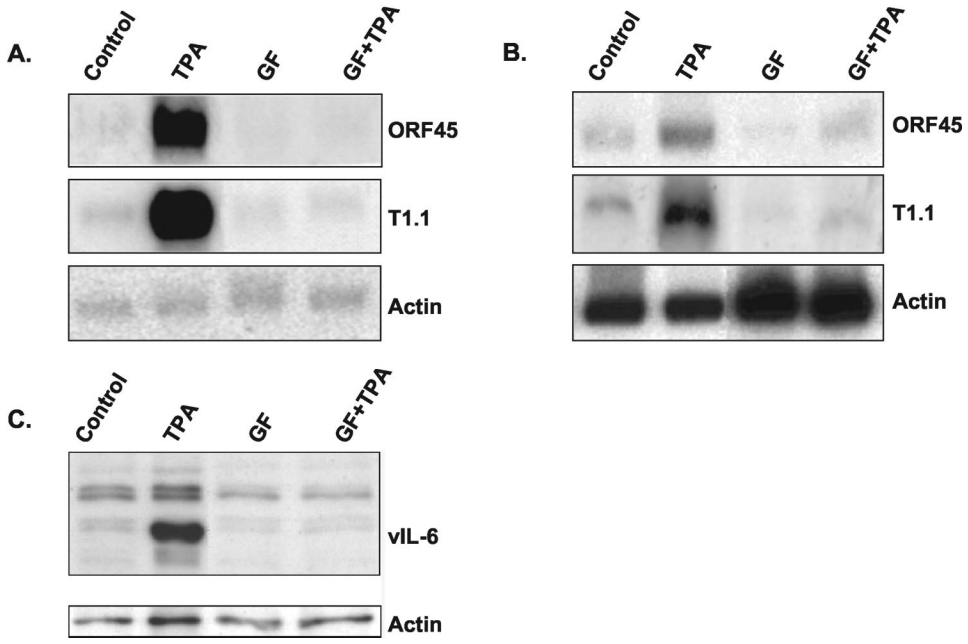


FIG. 1. Effect of TPA and inhibitor of PKC on KSHV reactivation. Northern blot hybridizations with T1.1 and KSHV/ORF45 probes of total RNA extracted from BCP-1 (A) and BCBL-1 (B) cells 24 h after treatment. Cells were subcultured at 2×10^5 cells per milliliter, incubated overnight, and exposed to 20 ng of TPA (Sigma Chemical Co., St Louis, Mo.)/ml or 5 μM GF 109203X (Calbiochem, San Diego, Calif.) for 24 h or exposed to 5 μM GF 109203X for 30 min before the addition of TPA for 24 h. Untreated cells were used as controls. The GAPDH transcript was analyzed as a control for equal RNA loading. Protein extracts were prepared from BCP-1 cells, and equal amounts of protein (30 μg) were loaded per lane. Following sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transfer of proteins to nitrocellulose, blots were probed for vIL-6 by Western blot analysis. Actin antibody was used to control for equal loading (C). The results shown are representative of those from three similar experiments.

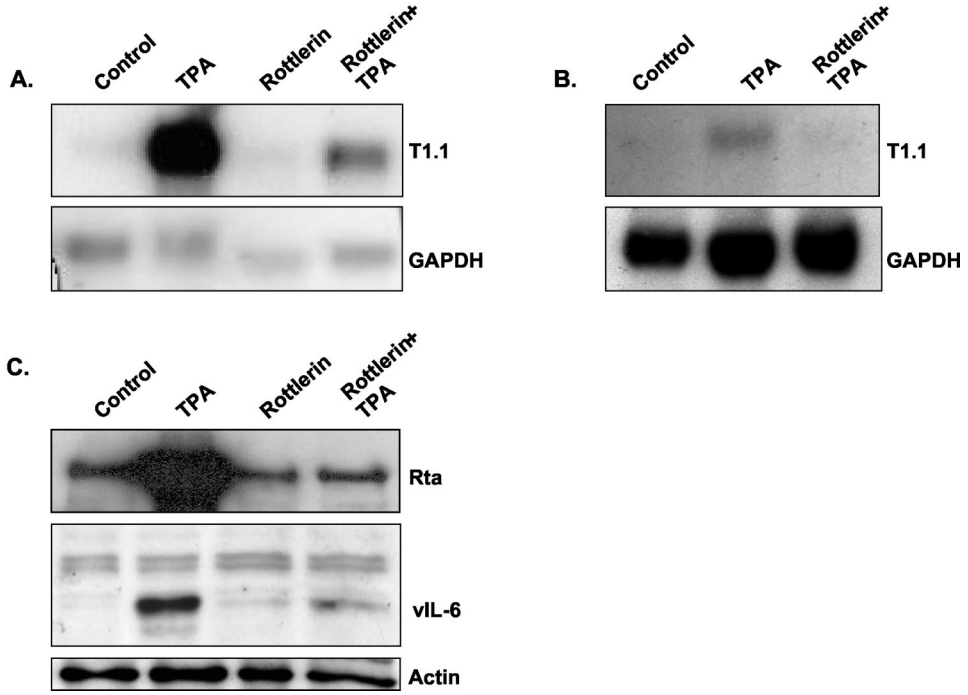


FIG. 2. Effect of the PKCδ inhibitor rottlerin on TPA-dependent virus lytic induction. Cells were pretreated with 5 μM rottlerin (Calbiochem) for 30 min followed by 24 h of treatment with 20 ng of TPA/ml. RNA extracts from BCP-1 (A) and BCBL-1 (B) cells were then analyzed for the T1.1 early transcript by Northern blot hybridization, and protein extracts from BCP-1 cells were assayed for the expression of KSHV/Rta and vIL-6 by Western blot analysis (C). As shown, TPA induced virus reactivation, whereas pretreatment with rottlerin inhibited the TPA-induced virus reactivation. The results shown are representative of those from three similar experiments.

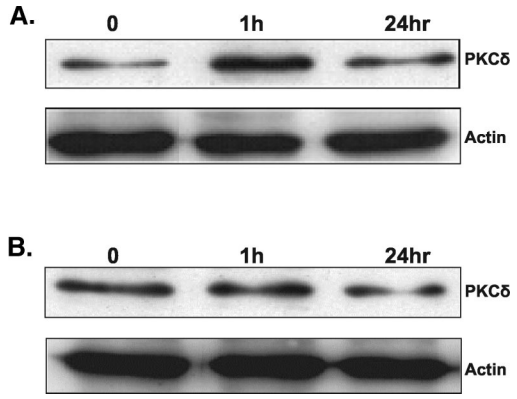
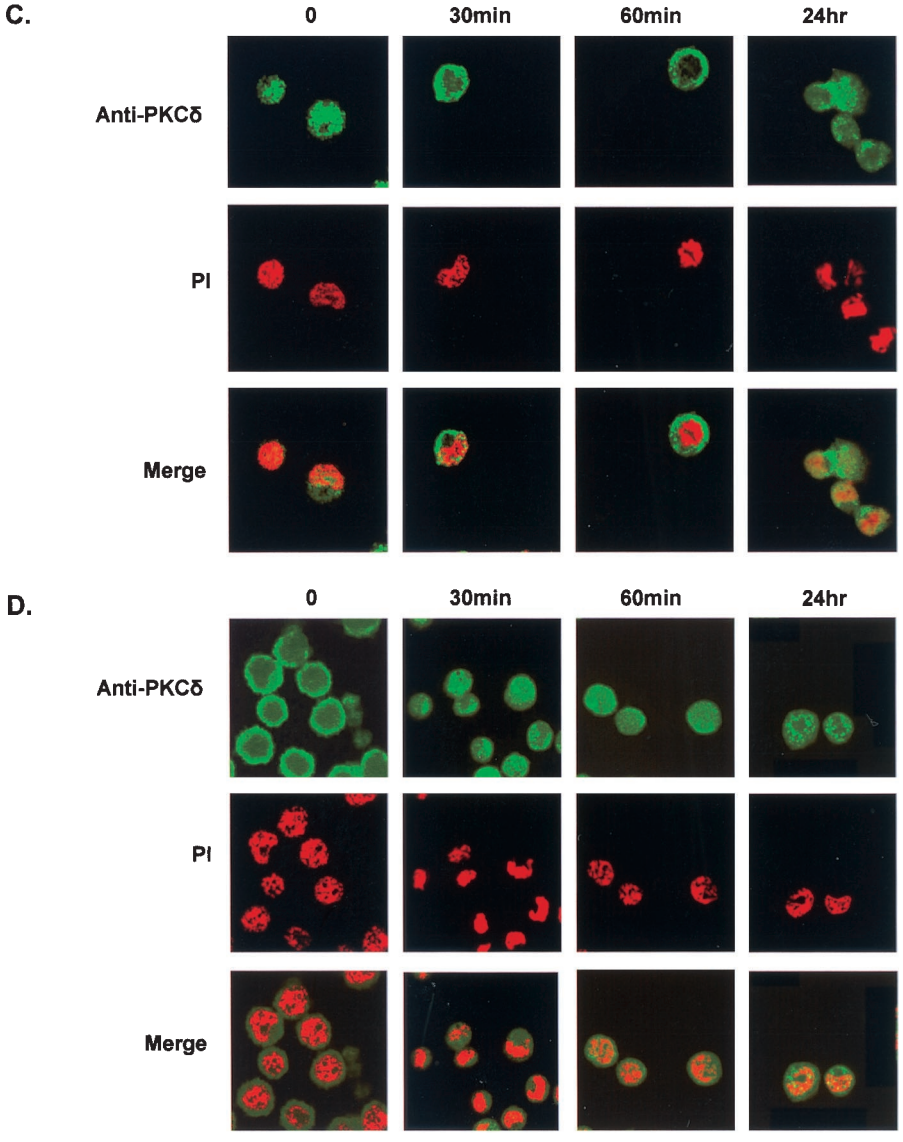


FIG. 3. Expression and translocation of PKCδ in BCP-1 and BCBL-1 cells that were treated with TPA. The expression of the PKCδ isoform was examined by using anti-human PKCδ (nPKCδ C-20; Santa Cruz) rabbit polyclonal immunoglobulin G in protein extracts from BCP-1 (A) and BCBL-1 (B) cells growing under standard growth conditions and from cells that were treated with TPA for 30 min, 60 min, and 24 h. The membrane was then probed with antiactin antibody. Fixed BCP-1 (C) and BCBL-1 (D) cells were incubated with rabbit anti-PKCδ antibody followed by an anti-rabbit antibody conjugated to fluorescein isothiocyanate. Propidium iodide (PI) staining was used to mark nuclei. Cells were visualized by confocal microscopy (Bio-Rad MRC 1024 confocal scan head mounted on a Nikon microscope). The results are from one of three similar experiments.



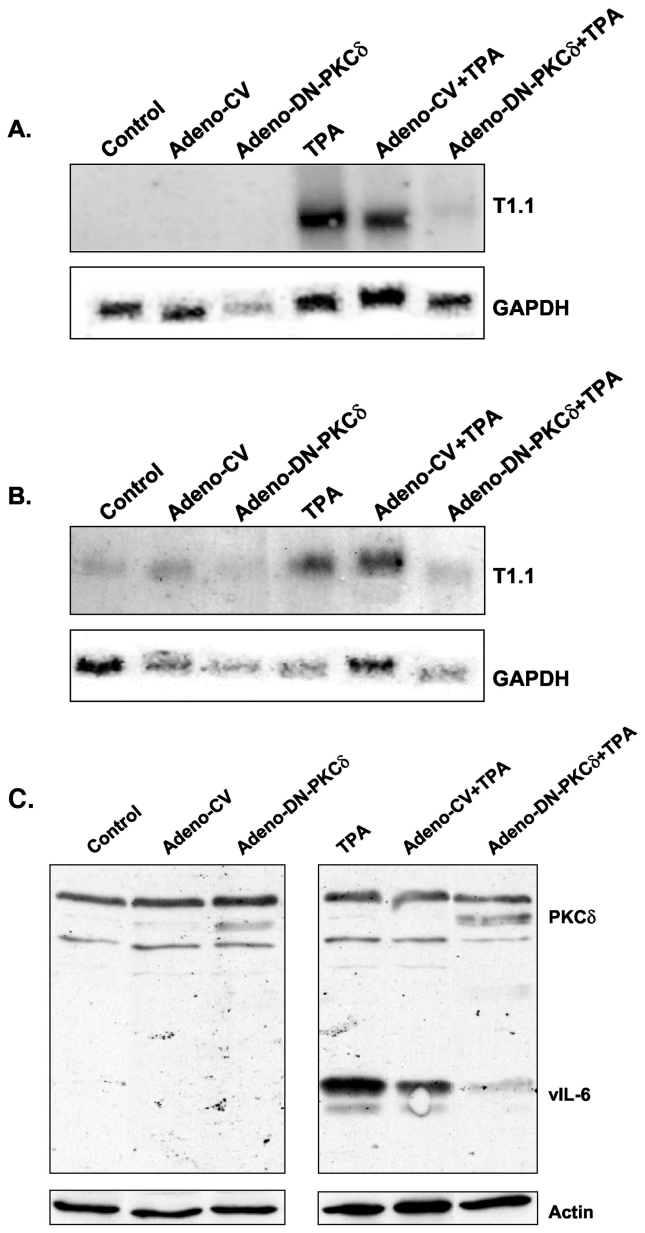


FIG. 4. Dominant-negative PKCδ expressed by an adenovirus vector inhibits TPA-mediated KSHV reactivation. Cells were infected with a recombinant adenovirus vector that expresses dominant-negative PKCδ (Adeno-DN-PKCδ). Twenty-four hours after the adenoviral transduction, cells were either treated with TPA or left untouched. RNA extracts from BCP-1 (A) and BCBL-1 (B) cells were then analyzed for the T1.1 early transcript. Expression of the ectopically expressed mouse dominant-negative PKCδ and vIL-6 was monitored in BCP-1 cells by Western blot analysis 24 h after the addition of TPA (C). Infection with empty adenovirus vector (Adeno-CV) was used as a control. Actin antibody was used to control for equal loading. The results shown are representative of those from three similar experiments.

This was evident by the induction of the expression of the immediate-early KSHV/ORF45 transcript (66), the T1.1 early transcript (65), and the early lytic protein viral IL-6 (vIL-6) (38) 24 h after stimulation (Fig. 1). Inhibition of the TPA-mediated virus reactivation was evident when 5 μM GF 109203X (bisindolylmaleimide I) (56), which inhibits the PKC α, β, γ, δ, and ε isoforms (31), was added 30 min prior to the addition of TPA.

To further evaluate the role of PKC in TPA stimulation of KSHV reactivation, we treated the cells with 5 μM rottlerin, a selective inhibitor of PKCδ (24). Results shown in Fig. 2 demonstrate that rottlerin largely reduced the TPA-dependent induction of KSHV in BCP-1 and BCBL-1 cells, suggesting an essential role for PKCδ activity in virus reactivation. Of note, we monitored possible toxic effects of the pharmacological treatments by cell cycle analysis with a fluorescence-activated cell sorter and found that treatment with rottlerin alone induced high levels of cell death in BCBL-1 but not in BCP-1 cells, whereas combined treatment with rottlerin and TPA avoided this response (data not shown). This effect probably reflects the nonspecific activity of rottlerin.

Expression and translocation of PKCδ prior to and after the addition of TPA. To further study the possible involvement of the PKCδ isoform in TPA-induced lytic reactivation of KSHV, we examined the effect of TPA stimulation on the expression and translocation of PKCδ. These experiments were necessary since prolonged exposure to TPA is known to induce down-regulation of the classical and novel PKC isoforms (45) and translocation of PKC is characteristic of PKC activation (6, 45). We detected expression of PKCδ in both cell lines (Fig. 3A and B) while an elevated level of expression was noted in BCP-1 cells 1 h after TPA stimulation. The cellular localization varied between cell lines, yet transient translocation of PKCδ was evident upon TPA stimulation both in BCP-1 and BCBL-1 cells (Fig. 3C and D).

Ectopic expression of dominant-negative PKCδ inhibits TPA-mediated KSHV reactivation. Though rottlerin has been widely used to study the role of PKCδ (14, 34, 64), some questions about the use of this compound have been raised recently (15, 30, 35, 54). Therefore, we further explored the role of PKCδ in virus lytic reactivation by employing recombinant adenoviral vectors (28) to transiently overexpress a mouse kinase-defective K376R PKCδ mutant (4). Overexpression of the transduced gene was confirmed by Western blot analysis with antibodies to the PKCδ that barely recognize the human isoform (nPKCδ rabbit polyclonal immunoglobulin G; Santa Cruz Biotechnology, Inc.). In accord with the findings obtained with rottlerin, expression of the dominant-negative PKCδ mutant largely inhibited KSHV lytic reactivation (Fig. 4). This result is consistent with the hypothesis that KSHV lytic reactivation by TPA depends to a large extent on the activity of PKCδ.

Ectopic expression of PKCδ does not affect KSHV lytic reactivation. Based on the findings that inhibition of PKCδ activity by rottlerin or by ectopic expression of the kinase-inactive PKCδ inhibited TPA-mediated KSHV lytic reactivation, we further investigated the role of PKCδ activation in KSHV lytic reactivation. We transduced the PKCδ with a recombinant adenovirus and assayed its effect on virus reactivation in the absence of and following the addition of TPA. Ectopic expression of PKCδ did not induce virus reactivation nor synergize with TPA in the induction of lytic KSHV reactivation. Similar results were obtained with bistratene A, a

cyclic polyether toxin that activates PKCδ (23, 58–60) (data not shown).

Taken together, our data suggest the following: (i) PKC is an important mediator in regulating KSHV lytic reactivation after TPA stimulation, (ii) activation of PKCδ is essential for TPA-mediated KSHV lytic reactivation, and (iii) stimulation of PKCδ is not sufficient to induce KSHV lytic reactivation. Our experiments suggest that non-PKC phorbol ester receptors, such as RasGRP and chimaerins, probably do not play a primary role in TPA-mediated virus reactivation; however, this pathway could have a secondary role that has not been explored. Notably, we observed translocation of PKCδ in the majority of cells that were treated with TPA, though virus activation occurs only in a small fraction of the cells (63). This implies that additional cellular molecules may act as rate-limiting factors for virus reactivation. It is also reasonable to assume that methylations, deletions, or rearrangements of key genes on the KSHV genome prevent KSHV reactivation in a subset of cells regardless of the cellular condition. Downstream effectors of PKC in this pathway have yet to be identified. Since PKC activation frequently leads to activation of members of the mitogen-activated protein kinases that can also be activated in response to a variety of extracellular stimuli and stress, one may envision a number of alternative signal transducing pathways that could induce lytic KSHV reactivation. In addition, isoforms of PKC may posttranslationally modulate the DNA-binding and transcriptional activity of KSHV/Rta.

Emerging evidence points to central roles for PKC isoforms during various phases of infection with different viruses. Activation of PKCζ during primary de novo infection has been recently reported to play an essential role during the initial stages of KSHV infection (42). Similarly, the entry of several other enveloped viruses, including rhabdoviruses, alphaviruses, poxviruses, adenoviruses, and influenza virus, has been proposed to require the activity of PKC (13, 50). Enhancer activation of the human immunodeficiency virus provirus is affected by PKC (20), and the use of synthetic analogues of DAG in conjunction with highly active antiretroviral therapy has been recently proposed (25). Infection with murine cytomegalovirus has been shown to recruit cellular PKC for phosphorylation and dissolution of the nuclear lamina (40). Alternatively, during infection, viruses may target PKC isoforms, which may in turn alter the natural functions of the infected cells (3, 53, 68). Thus, the variable effects of PKC on a range of signal transduction pathways may alter the outcomes of virus exposure and infection both in vitro and in vivo. This may also provide, in the future, a potential therapeutic means to interfere with the consequence of virus infection. As the distinct characteristics attributed to the various PKC isoforms suggest that the composition of PKC isoforms in a particular cell type should determine its cellular response, extensive exploration of the involvement of PKC in KSHV lytic reactivation in a variety of cell types is necessary.

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