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Review Article

The interaction between the Wnt/β-catenin signaling cascade and PKG activation in cancer

Kevin Lee✉, Gary A Piazza

Drug Discovery Research Center, Mitchell Cancer Institute, University of South Alabama, Mobile, AL 36604-1405, USA.

Abstract

The activation of the Wnt/β-catenin signaling cascade has been well studied and documented in colorectal cancer (CRC). The long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) has been shown to reduce the incidence and risk of death from CRC in numerous epidemiological studies. The NSAID sulindac has also been reported to cause regression of precancerous adenomas in individuals with familial adenomatous polyposis who are at high risk of developing CRC. The mechanism responsible for cancer chemopreventive activity of NSAIDs is not well understood but may be unrelated to their cyclooxygenase inhibitory activity. Emerging evidence suggests that sulindac inhibits the growth of colon tumor cells by suppressing the activity of certain phosphodiesterase isozymes to activate cGMP-dependent protein kinase, PKG, through the elevation of the second messenger cyclic guanosine monophosphote, cGMP. PKG activation has been shown to inhibit the nuclear translocation of β-catenin, reduce β-catenin mRNA and protein levels, and suppress the transcriptional activity of β-catenin. This review describes the relationship between the Wnt/β-catenin signaling cascade and the activation of PKG through PDE inhibition and elevation of intracellular cGMP levels.

Keywords: Wnt, β-catenin, PKG, cGMP, PDE, NSAID, colon cancer, breast cancer

Introduction

Colorectal cancer (CRC) is the third most common type of cancer among men and women in the United States. It is estimated that over 71,000 men and 65,000 women were diagnosed with CRC and over 26,000 men and 24,000 women died of this malignancy in 2014[1]. CRC is a disease that can take decades to develop in humans that requires sequential genetic mutations to tumor suppressor or oncogenes, including *APC*, *KRAS*, *PIK3CA*, *SMAD4,* and *TP53*[2]. Even though early detection procedures have been shown to decrease mortality and incidence of CRC, there is an unmet medical need for safe and effective drugs to use for CRC for chemoprevention, especially in individuals at high risk of developing CRC[3].

The Wnt proteins constitute a major family of proteins that are involved in many biologic processes including proliferation as well as embryological development[4]. Once Wnt is translated, it is further processed by the endoplasmic reticulum (ER) and the Golgi to be secreted into the extracellular space[4]. In the extracellular environment, Wnt can then participate in short range paracrine signaling where it binds the Frizzled

✉Corresponding author: Kevin Lee, Drug Discovery Research Center, Mitchell Cancer Institute, University of South Alabama, Mobile, AL 36604-1405, USA. Tel: 1-251-445-9885, E-mail: kjlee@health.southalabama.edu.

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receptor on nearby cell membranes[5]. Under conditions in which Wnt is absent, β-catenin that is not bound at cadherin junctions (free β-catenin) associates with adenomatous polyposis coli (APC), Axin, and glycogen synthase kinase 3β (GSK3β) in a complex known as the destruction complex. Upon association with the destruction complex, GSK3β phosphorylates β-catenin[6], thus priming it for ubiquitination by β-TRCP and degradation by the proteasome[7]. Wnt binding to Frizzled and its co-receptor, low-density lipoprotein receptor-related protein (LRP), induces phosphorylation of Dishevelled (Dvl)[8]. Phosphorylated Dvl then associates with Axin, inducing dissociation of the β-catenin destruction complex. Free β-catenin then accumulates in the cytoplasm of the cell and is translocated to the nucleus. Once in the nucleus β-catenin binds to T cell factor/lymphoid-enhancer factor (Tcf/Lef) transcription factors and activates the transcription of several target genes, which can include *c-Myc*, *cyclin D1*, *gastrin*, and *ITF-2* (***Fig. 1***)[9–14].

Disregulation of Wnt signaling in cancer

Genetic mutations in components that make up the Wnt signaling pathway can occur in as much as 80% of CRC[15]. Disruptions in the β-catenin destruction complex can result in activation of the Wnt signaling pathway that could lead to aberrant signaling through the Tcf/Lef transcription factors. APC has been shown to act as a tumor suppressor whereby it is known to suppress cytoplasmic and nuclear levels of β-catenin in CRC cells[16]. Indeed, APC has been referred to as one of the gatekeepers of colorectal tumorigenesis[2]. Studies have shown that mutations yielding a truncated APC protein are responsible for patients with familial adenomatous polyposis (FAP). A mouse model has been shown to harbor a single T-A transversion at codon 850 in the *APC* gene which results in a nonsense mutation[17]. It should be noted that mice with a homozygous loss of *APC* do not survive through the early stages of embryonic development[18]. These mice, known as $APC^{min/+}$ mice (multiple intestinal neoplasia) develop spontaneous tumors throughout the small intestine and the colon, and has been used as a useful tool in the study of CRC. Inbreeding of a single mouse containg a large number of colorectal tumors has yielded a strain that produces large amounts of colorectal tumors thereby improving their usefulness as a mouse model in the study of CRC and for assessing the efficacy of chemopreventive drugs for CRC[19].

Mutations in other components of the Wnt signaling pathway have also been shown to play a role in colon tumorigenesis. Mutations at the GSK3β regulatory sites in the β-catenin gene, *CTNNB1*, prevents phosphorylation by GSK3β, thereby allowing β-catenin to bypass the destruction complex[20-22]. These stabilizing muta-

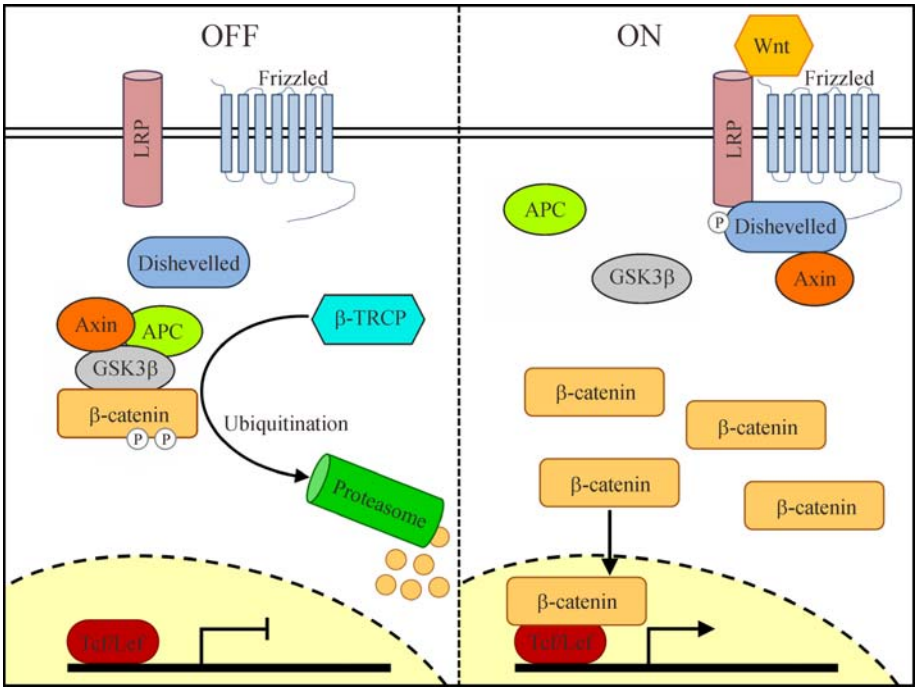

Fig. 1 **Wnt signaling.** In the off state, β-catenin is bound by the destruction complex, phosphorylated, ubiquitinated, and degraded. The presence of Wnt causes phosphorylation of disheveled by LRP, and this recruits axin which disrupts the β-catenin destruction complex, thereby allowing β-catenin accumulation and translocation to the nucleus. In the nucleus, β-catenin binds to Tcf/Lef transcription factors to induce transcription of target genes.

tions induces an increase in β-catenin protein expression in humans[23]. In addition, a mutation in GSK3β has been shown to inhibit its ability to phosphorylate substrates, thereby allowing β-catenin to accumulate in the cells[24].

NSAIDs in cancer

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed to treat inflammatory conditions and are well known to inhibit cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) and suppress prostaglandin synthesis. Epidemiological studies have linked NSAIDs to a decrease in the incidence and the mortality rate of CRC as well as other malignancies[25]. The NSAID, sulindac has been shown to reduce the number of colorectal polyps as well as their mean diameter in patients with FAP, which is characterized by the formation of hundreds of adenomas that have the potential to develop into adenocarcinomas[26]. In *APC*$^{min/+}$ mice, sulindac significantly reduced the incidence of intestinal adenomas[27]. A metabolite of sulindac, sulindac sulfone, was also shown to inhibit tumorigenesis in the azoxymethane-induced rat model of colon tumorigenesis[28].

Sulindac can be metabolized by the liver into a sulfone metabolite through oxidation or into a sulfide metabolite through bioreduction involving colonic bacteria[29]. Reduction into sulindac sulfide is reversible and this metabolite is responsible for the anti-inflammatory mechanism of sulindac, while the sulfone metabolite lacks anti-inflammatory activity and does not inhibit cyclooxygenase. Interestingly, both sulindac sulfide and sulfone have been shown to inhibit tumor cell growth in vitro[28], while sulindac sulfone shows similar anticancer activity as sulindac in rodent models of chemical-induced tumorigenesis[28]. This suggests that a cyclooxygenase-independent mechanism is responsible for the cancer chemopreventive activity of sulindac, and possibly other NSAIDs. Sulindac sulfone was shown to be effective in clinical trials for individuals with FAP but was not approved by the FDA, likely attributed to low potency and a narrow therapeutic window in which hepatotoxicity was the dose-limiting toxicity[30].

Phosphodiesterase implications in cancer

Cyclic nucleotide degrading assays were first used to show that sulindac sulfone inhibits phosphodiesterase (PDE) activity at concentrations equivalent to those required to inhibit colon tumor cell growth[31]. Cyclic nucleotides are generated by enzymes known as cyclases that use GTP or ATP to generate cyclic-GMP or cyclic-AMP, respectively. The cyclic nucleotides are then hydrolyzed into 5′GMP or 5′AMP through the action of phosphodiesterases in which inhibitors can cause a transient or sustained elevation of intracellular cGMP or cAMP levels, respectively. There are currently 11 known families of PDE's that contain over 50 different isoforms or splice variants[32]. Phosphodiesterases 5, 6, and 9 are cGMP specific, meaning that they selectively act to hydrolyze cGMP into 5′GMP[32]. Phosphodiesterases 4, 7, and 8 are cAMP specific, meaning that they selectively hydrolyze cAMP into 5′AMP[32]. PDEs 1, 2, 3, 10, and 11 are dual substrate PDEs, meaning that they can hydrolyze both cGMP and cAMP into 5′GMP and 5′AMP, respectively[32]. Cyclic nucleotides can act as second messenger molecules in signaling pathways by activating protein kinases or other biochemical processes, including ion channels or regulating other PDE isozymes. In the case of protein kinases, cGMP can bind to and activate cyclic GMP-dependent protein kinase (PKG), while cyclic AMP can bind and activate cAMP-dependent protein kinase (PKA)[33]. Cyclic nucleotides can also influence nucleotide-gated ion channels, or bind to certain cyclic nucleotide dependent PDEs resulting in their activation[33]. There is also a level of crosstalk between cGMP and cAMP whereby the levels of cGMP can act to regulate the levels of cAMP through PDE activation or inhibition[34]. It has also been shown that cAMP can activate PKG in coronary myocytes, thereby introducing another level of cyclic nucleotide cross-activation[35].

Cyclic nucleotide elevation through PDE inhibition was shown to inhibit tumor cell growth in multiple cancer cell lines[11,36-38]. In those same studies, it was shown that certain PDEs (PDE5, and PDE10) were expressed in high levels in tumor cells when compared to normal cells of the same tissue origin and that when these phosphodiesterases were inhibited through either pharmacological mechanisms or through genetic silencing, cyclic nucleotide levels were increased leading to activation of protein kinases, PKG and PKA. Sulindac was shown in these studies to inhibit tumor cells through a cyclooxygenase-independent mechanism of cGMP PDE inhibition which led to the elevation of cGMP and thus the activation of PKG. It has also been shown that inhibition of PDE9 induced an increase in the concentration of intracellular cGMP in estrogen receptor (ER) positive breast cancer cell lines (MCF-7) as well as in ER negative breast cancer cell lines (MDA-MB-468)[39]. However, the authors did not determine any downstream targets other than caspase activation and subsequent apoptosis[39]. It has also been shown

that a PDE2 selective inhibitor induced cGMP elevation and attenuated UVB-induced carcinogenesis in mice[40]. Patients with chronic lymphocytic leukemia (CLL) were shown to have an increase in PDE7B expression[41]. Cells isolated from these patients underwent apoptosis when treated with a PDE7 inhibitor, albeit through an increase in cAMP[41]. PDE10 was shown to be inhibited through the highly selective PDE10 inhibitor, Pf-2545920 (MP-10), which also led to the elevation of cGMP and the activation of PKG in colon cancer cell lines[11-12]. The increase in activity of PKG was shown to have an effect on β-catenin signaling and thus initiation of apoptosis[11-12,37-38,42-44].

Interaction of PKG and the Wnt signaling cascade

PKG is a kinase that phosphorylates specific proteins depending on the intracellular level of cGMP. PKG is encoded by two distinct genes in eukaryotes; *prkg1* encodes PKG-I while *prkg2* encodes PKG-II. PKG-I further exists in two isofoms based on alternative splicing, PKG-Iα and PKG-Iβ. PKG consists of an N terminus comprised of a leucine zipper and an auto-inhibitory domain, a regulatory domain, and a catalytic domain which binds ATP to catalyze phosphate transfer to target molecules[45]. Both PKG-Iα and PKG-Iβ are found in vascular smooth muscle cells, the uterus, gastrointestinal tract, kidney, and trachea where they play a role in smooth muscle relaxation[46]. In the intestinal tract, PKG-II is involved in the activation of the cystic fibrosis transmembrane regulator (CFTR) anion channel and chloride channels (ClC), causing the efflux of chloride and bicarbonate. Water is then effluxed into the intestinal lumen[47]. Upon binding of cGMP to the regulatory domain, the auto-inhibitory domain of PKG is released allowing binding of ATP in the catalytic domain and subsequent phosphorylation of target proteins. PDEs therefore can regulate the activity of PKG due to their relative abundance or activity in cells, where high PDE levels or activity would keep cyclic nucleotides at low levels, while suppression of PDE levels or activity can increase the levels of cyclic nucleotides and activate protein kinases such as PKG or other downstream mediators.

Ectopic expression of PKG-I was shown to induce apoptosis and reduce cell migration of colon tumor cells[44]. These studies showed that expression of constitutively active forms of PKG-Iα, as well as PKG-Iβ reduced colony formation in SW-480 colon tumor cells. The authors also reported that overexpression of PKG-Iβ caused a reduction in levels of cyclin D1, as well as β-catenin and at the same time observed an increase in levels of $p21^{CIP1}$. In addition, the investigators noted that cyclin D1 was transcriptionally suppressed.

The COX-2 selective inhibitor, celecoxib, was also reported to inhibit the growth of SW-480 colon tumor cells by a COX-independent mechanism involving PDE inhibition[48]. In these studies, celecoxib increased intracellular levels of cGMP and activated PKG as shown by an increase in vasodilator-stimulated phosphoprotein (VASP) phosphorylation[49]. Furthermore, the sulfone metabolite of sulindac (exisulind) was shown to inhibit PDE5 at lower concentrations than those required to inhibit tumor cell growth and induce apoptosis[31]. These same studies also showed that PKG was activated upon treatment with sulindac sulfone, which corresponded with a decrease in β-catenin, as well as a reduction in cyclin D1 levels.

The sulfide metabolite of sulindac was shown to inhibit the growth of breast cancer cell lines and induce apoptosis at the same concentrations by which treatment increased intracellular cGMP levels[36]. This was also accompanied by an increase in the phosphorylation of VASP at serine 239. However, a guanylyl cyclase inhibitor caused an increase in the concentration of sulindac sulfide required to inhibit growth. The authors also noted that treatment with an adenylyl cyclase activator, forskolin, had little to no effect on breast tumor cell growth. They concluded that sulindac sulfide, through the inhibition of PDE5, caused cell cycle arrest and induced apoptosis through cGMP elevation, and activation of PKG. It was further shown that sulindac sulfide selectively induced apoptosis in breast tumor cells, while normal mammary epithelial cells were insensitive, through the redction in nuclear translocation of β-catenin[37]. Silencing of PDE5 by siRNA inhibited the growth of breast tumor cells and suppressed β-catenin levels[37].

Sulindac sulfide was also shown to inhibit the growth of colon tumor cell lines selectively over normal colon epithelial cells as well as an increase in caspase activity and a reduction in proliferation accompanied by an increase in intracellular cGMP[50]. Consistent with a mechanism involving cGMP PDE inhibition, PDE5 was shown to be overexpressed in colon tumor cells compared to normal colon epithelial cells. Upon silencing of PDE5 with siRNA, cell viability was decreased as well as proliferation; conversely, caspase activity was increased. An analog of cGMP, 8-Bromo-cGMP, was used to show that β-catenin levels are reduced, as well as cyclin D1 and survivin, with PKG activation. The reduction in β-catenin in response to sulindac sulfide treatment was shown to be at the transcription level through the use of a Tcf/Lef reporter

assay driving the CTNNB1 promoter. Furthermore, a non-COX inhibitory derivative of sulindac more potently inhibited the growth of colon tumor cell lines than sulindac sulfide[38]. This novel sulindac derivative more potently and selectively inhibited PDE5 over other PDEs and inhibited tumor cell growth through similar mechanisms.

Further research into cGMP PDEs revealed that PDE10 mRNA and protein are overexpressed in colon tumor cell lines compared to normal colon epithelial cells[11]. PDE10 is also overexpressed in human colon tumors and in tumors from *APC*$^{min/+}$ mice relative to normal colon mucosa. PDE10 inhibitors suppressed the proliferation and induced apoptosis of colon tumor cells by a mechanism involving G1 cell cycle arrest. Knockdown of PDE10 yielded similar effects as well as causing a decrease in cyclin D1 and survivin. Other studies implicated β-catenin in the PDE10 signaling pathway by showing that knockdown of PDE10 caused a reduction in β-catenin transcriptional activity using a Tcf/Lef reporter assay[11]. The highly selective and potent PDE10 inhibitor, Pf-2545920 (MP-10), was also shown to induce apoptosis through an increase in intracellular cGMP, the activation of PKG, and the reduction of β-catenin translocation to the nucleus[12]. As previously mentioned, PDE10 is a dual-specific phosphodiesterase capable of hydrolyzing both cGMP and cAMP. Therefore, further studies are necessary to determine if the effects of PDE10 inhibition involve an increase in cGMP or cAMP or an increase in both. Through the use of pharmacological inhibitors of PKG and PKA, recent studies suggest that the anticancer activity resulting from PDE10 inhibition is exclusively through the activity of cGMP/PKG signaling in which the effects on cAMP/PKA signaling may be ancillary. As mentioned previously, the inhibition of the cGMP specific PDE5 similarly led to a reduction of β-catenin, survivin, cyclin D1 levels, as well as a reduction in Tcf/Lef transcriptional activity[37,50]. This supports the possibility that the effects of PDE10 inhibition exclusively involve cGMP/PKG signaling. In addition, dual inhibition of PDE5 and PDE10 results in additive or synergistic effects on cGMP/PKG signaling on β-catenin mediated transcriptional activity[51].

Further evidence suggests that NSAIDs can inhibit Wnt/β-catenin signaling in prostate cancer cell lines[52]. The investigators determined that sulindac sulfide inhibits tumor cell growth through a β-catenin mechanism by utilizing a novel small molecule designed to inhibit the interaction of β-catenin with Tcf/Lef[53]. However, the authors did not investigate if the effects were due to the activation of PKG or through some other mechanism.

Ectopic expression of PKG-Iβ was shown to cause a decrease in transcriptional activity of the *CTNNB1* gene causing a decrease in β-catenin expression but not through an increase in degradation[42]. Activation of JNK occurred concomitantly with a decrease in

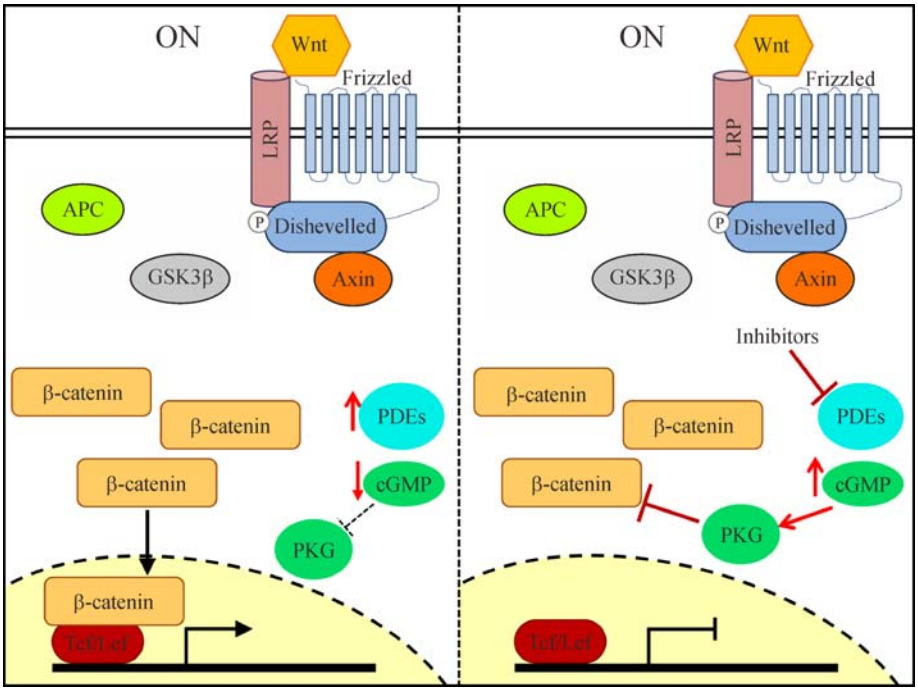

Fig. 2 **Phosphodiesterase activity.** The increased activity of PDEs (left) causes the intracellular concentration of cGMP to remain at low levels, thereby keeping the basal activity of PKG low. This allows β-catenin to translocate to the nucleus. When PDEs are inhibited by small molecules or through genetic silencing, the concentration of cGMP increases. The cyclic nucleotide then binds and activates PKG which inhibits β-catenin mediated signaling.

CTNNB1 transcription causing an increase in FOXO4 activity. FOXO has been shown to repress Wnt/β-catenin signaling through the decrease in β-catenin transcription in osteosarcoma cells[54]. This dual role of PKG in regards to a decrease in transcriptional activity has been limited to the PKG-I isoform; therefore, more studies would need to be conducted to determine if these findings translate to PKG-II.

Discussion

The Wnt/β-catenin signaling cascade has been implicated in many biologic processes including, but not limited to cell growth, differentiation, development, as well as even cancer. Indeed, the APC protein is one of the best known examples of a tumor suppressor that regulates β-catenin signaling. NSAIDs are known to be effective for the treatment of patients with FAP but are not FDA approved for such a use due to potentially fatal side effects relating to their cyclooxygenase inhibitory activity and suppression of physiologically important prostaglandins. The reduction of incidence of adenomas in $APC^{min/+}$ mice treated with sulindac suggested that sulindac played a role in modulating Wnt/β-catenin signaling. It was later discovered that the inhibition of PDE5 was responsible for a decrease in Wnt/β-catenin signaling through the activation of PKG. In addition, PDE10 inhibition also resulted in an activation of PKG and a reduction in β-catenin mediated signaling. More research would need to be taken in order to ensure results seen in regard to PDE10 inhibition were due to PKG activation. In summary, the activation of PKG has been shown to modulate the Wnt/β-catenin signaling cascade through the reduction of β-catenin protein levels, blocking the nuclear translocation of β-catenin, decreasing the level of β-catenin transcriptional activity through the Tcf/Lef promoter, decreasing the transcription of β-catenin itself, or through interactions with other transcription factors. A depiction of how PDEs interact with the Wnt/β-catenin signaling cascade is shown in ***Fig. 2***.

However, even though great strides have been made in understanding the role PKG plays in the Wnt/β-catenin signaling cascade, the exact mechanism through which PKG exerts its effects have not yet been fully elucidated. More research into this area will help guide the research community in understanding the role that PKG plays in modulating Wnt/β-catenin signaling.

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