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Wnt Signaling in Hematopoiesis: Crucial Factors for Self-Renewal, Proliferation, and Cell fate Decisions

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ABSTRACT

A large number of studies from many different laboratories have implicated the Wnt signaling pathway in regulation of hematopoiesis. However, different inducible gain- and loss-of-function approaches yielded controversial and some times contradictory results. In this prospect we will review the current ideas on Wnt signaling in hematopoiesis and early lymphopoiesis. Reviewing this large body of knowledge let us to hypothesize that different levels of activation of the pathway, dosages of Wnt signaling required and the interference by other signals in the context of Wnt activation collectively explain these controversies. Besides differences in dosage, differences in biological function of Wnt proteins in various blood cell types also is a major factor to take into account. Our own work has shown that while in the thymus Wnt signaling provides cytokine-like, proliferative stimuli to developing thymocytes, canonical Wnt signaling in HSC regulates cell fate decisions, in particular self-renewal versus differentiation. J. Cell. Biochem. 109: 844–849, 2010.

KEY WORDS: Wnt; HEMATOPOIESIS; T CELL DEVELOPMENT

Hematopoiesis is a continuous process in which stem/progenitor cells develop into mature blood cells. Hematopoietic stem cells (HSCs) are self-renewing, multipotent progenitors that give rise to all types of mature blood cells. HSC are normally very rare and are thought to spend most of their time in a quiescent state while residing in specialized stromal cell containing niches [Wilson et al., 2007]. The bone marrow is the major site of adult hematopoiesis, but HSCs can also undergo hematopoiesis in the spleen and liver during periods of hematopoietic stress, while the liver also is the major hematopoietic organ in fetal life [Wilson and Trumpp, 2006]. Through mechanisms that are only partially understood, the integrity of stem cells is maintained throughout life. That is, they maintain competence to self-renew and to generate progenitors capable of making billions of blood cells each day. HSCs are thought to reside within HSC niches, which are specialized microenvironments within hematopoietic tissues created by supporting cells that express membrane-bound and secreted factors that promote HSC survival and self-renewal, and that regulate HSC migration and differentiation [Wilson and Trumpp, 2006]. A plethora of pathways is involved in HSC biology in the niche, among which Wnt, Notch, BMP, TGFβ/SMAD, FGF, JAK/STTAT and several others [reviewed in Blank et al., 2008]. Here we will confine ourselves to the Wnt pathway, but note that several controversial outcomes on the role of Wnt signaling in hematopoiesis may have to do with cross talk between these pathways that regulate HSC maintenance.

THE Wnt SIGNALING PATHWAY

In total 19 different WNT proteins have been identified in both human and mouse genomes and their patterns of expression overlap both spatially and temporally during development, raising the possibility of functional redundancy between WNT proteins [Nusse and Varmus, 1992]. At least three different Wnt pathways are currently recognized: the canonical Wnt pathway, which is mediated via β-catenin and Tcf/Lef factors; the planar cell polarity (PCP) pathway; and the Wnt-Ca^{2+} pathway [reviewed in Staal et al., 2008]. A large body of evidence [reviewed in, for instance, Malhotra and Kincade, 2009; Staal and Clevers, 2003; Staal et al., 2008] has shown that canonical Wnt signaling is essential for thymocyte proliferation and T cell development and most investigators are convinced that Wnt signaling is essential for HSC biology (see below). We will confine ourselves to the canonical Wnt pathway in this review, as the vast majority of hematopoietic studies involve

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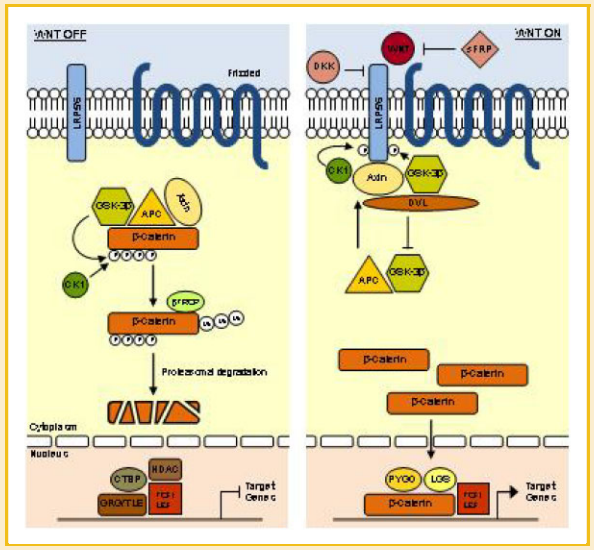

Fig. 1. The WNT signaling pathway. A simplified scheme is shown in the absence (OFF) or presence (ON) of Wnt signals. For details see text.

canonical Wnt signaling and only occasionally will remark on non-canonical Wnts.

Wnt proteins bind to their receptors, named Frizzled (Fz), thereby preventing proteosomal degradation of the Wnt mediator β-catenin (Fig. 1). Subsequently, β-catenin is translocated to the nucleus where it will form an active transcription complex with one of the members of the transcription factors downstream of the Wnt pathway: Tcf1 (T cell factor 1), Tcf3, Tcf4, or Lef1 (lymphocyte-enhancer-binding factor) [Okamura et al., 1998]. Tcf1 is mainly expressed in T lymphocytes, but also in HSC, TCF4 is widely expressed, also in the stem cells of gut and breast and Tcf3 is expressed in many embryonic tissues, including ES cells. Lef1 also is expressed in HSC, B lymphocytes and several other non-hematopoietic cell types.

In absence of a Wnt ligand binding to its receptor complex, β-catenin is broken down in the destruction complex composed of the scaffolding and tumor suppressor proteins APC and Axin and the CK1 and GSK-3β Ser/Thr kinases (Fig. 1). Binding of Wnt proteins to Fz/LRP receptor complex can be actively prevented by naturally occurring soluble decoy receptors such as sFRP, WIF, and DKK that binds and blocks the LRP5/6 coreceptor. Beta-catenin is phosphorylated in the destruction complex first on S45 by CK1, subsequently by GSK-3β on S33, S37, and T41 to create recognition sites for β-Trcp leading to ubiquitination and proteasomal breakdown [Aberle et al., 1997]. In the nucleus Tcf assembles a transcriptional repressor complex to silence Wnt target genes via recruitment of Groucho/TLE [Roose et al., 1998], CtBP and histone deacetylases (HDACs).

Upon Wnt binding to its receptor, a complex signaling cascade is initiated culminating in activation of Wnt target genes. These target genes have remained largely elusive in the hematopoietic system (in contrast to other tissues where Wnt target genes are largely known), with the exception of immature thymocytes where proliferation, cell adhesion, and anti-apoptotic genes have been identified as Wnt targets [Staal et al., 2004]. Formation of the Fz/LRP5/6 complex via Dlv (mammalian homologue of Drosophila disheveled) promotes phosphorylation of LRP5/6 by CK1 and GSK-3β, the very same kinases involved in β-catenin phosphorylation in the destruction complex [Zeng et al., 2008]. At the plasma membrane however, phosphorylation of LRP allows docking of Axin by binding to these phosphorylated residues thereby Axin is "pulled away" from the destruction complex [Zeng et al., 2005]. Indeed LRP6, Dvl, FZ, Axin, and GSK-3β all co localize to signalosomes enriched in lipid rafts and are internalized via caveolins [Yamamoto et al., 2006]. Thus, a membrane-associated form of GSK-3β, in contrast with cytosolic GSK-3β, stimulates Wnt signaling [Zeng et al., 2005]. Dimerization of Axin is crucial for signaling [Macdonald et al., 2007]. The recruitment of Axin to the plasma membrane leads to inhibition of β-catenin phosphorylation and halts its degradation via inhibition of the GSK-3β pool associated with the destruction complex. The accumulation of β-catenin in its N-terminally dephosphorylated forms [Staal et al., 2002] leads to its translocation to the nucleus. In the nucleus β-catenin binds to Tcf/Lef factors and recruits transcriptional activator cofactors such as LgS (legless, also known as Bcl9) and Pygopus, CBP/p300, Brahma, Med12/mediator and others to initiate transcription [Valenta et al., 2003; Townsley et al., 2004; Yasuda et al., 2004; Weiske and Huber, 2005; Li et al., 2007] and RNA elongation. In contrast, ICAT can inhibit interaction of β-catenin and Tcf/Lef thereby preventing assembly of the active bipartite transcription factor complex [Daniels and Weis, 2002].

GAIN-OF-FUNCTION STUDIES ON Wnt SIGNALING IN HSC

WNT proteins were for the first time discovered as normal growth/differentiation factors for hematopoietic progenitors in in vitro assays (Austin et al., 1997, Blood; Van Den Berg et al., 1998, Blood). However further investigation showed that Wnts could have a role in maintaining or inducing an undifferentiated phenotype in hematopoietic cells. Seminal work by Weissman and coworkers, using TCF-GFP reporters, demonstrated that Wnt–β-catenin-TCF/LEF signaling was active in HSCs [Rattis et al., 2004; Reya and Clevers, 2005]. Wnt signaling was also documented in HSCs after stimulation in vitro with purified Wnt3a [Reya et al., 2003; Willert et al., 2003]. Retroviral expression of a constitutively active form of β-catenin in murine Bcl2 transgenic HSCs led to an increase in the numbers of HSCs with enhanced ability to reconstitute lethally irradiated recipient mice [Reya et al., 2003; Willert et al., 2003]. Conversely, retroviral expression of the Wnt-signaling inhibitor Axin in the same system showed reduced reconstitution. These gain-of-function studies supported a functional role for Wnt–β-catenin–TCF pathway in hematopoiesis

However, these findings have come under scrutiny due to different results reported in papers from the laboratories of Achim Leutz and Claus Nerlov. These studies, also using a gain-of-function approach, showed that constitutive activation of β-catenin impaired multilineage differentiation and caused exhaustion of the HSC pool [Baba et al., 2005, 2006; Kirstetter et al., 2006; Scheller et al., 2006]. How can such different outcomes be explained? In part because of

different systems used: Reya et al. using retroviral vectors to over express a stabilized form of β-catenin in BCL-2 transgenic mice. The other two reports [Kirstetter et al., 2006; Scheller et al., 2006] used a transgenic approach to express a constitutively active form of β-catenin in all stem cells. Here a block in differentiation was observed, forced entry into the cell cycle leading to a transient expansion and consequently to exhaustion of long-term HSC as demonstrated by failure to reconstitute lethally irradiated mice. Since the first studies performed by Reya and coworkers were done using a BCL-2 transgenic background, this may partially explain the differences observed with the different approaches, underlying the complex interactions of Wnt signaling with other pathways. Also, a retrovirus such as used by Reya et al. [2003] is subject to methylation of promoter sequences in the LTR, therefore not all HSC and their progeny are likely to maintain high levels of β-catenin and therefore canonical Wnt signaling. Therefore, the differences between these studies could in part be explained by the methods used to over express β-catenin, leading to high and sometimes very high levels of Wnt signaling in HSC. Clearly very high Wnt signaling can be too much of a good thing. On the other hand it should be noted that two reports from Paul Kincade's laboratory could not readily confirm the first claims using similar retroviral vectors to express stabilized β-catenin in normal HSC [Baba et al., 2005, 2006]. These studies showed that expression of constitutively active β-catenin confers multilineage differentiation potential on lymphoid and myeloid progenitors. Recent work has shown that the more subtle approach of in vitro stimulation of HSC with recombinant Wnt3a lead to the dedifferentiation of committed B cells to more stem cell like cells, while purified murine HSC could be expanded roughly twofold [Malhotra et al., 2008]. In the same study the non-canonical Wnt5a protein had opposite effects.

LOSS-OF-FUNCTION STUDIES ON Wnt SIGNALING IN HSC

At least three studies with loss-of-function of the Wnt pathway provide evidence for a functional role of canonical Wnt signaling in hematopoiesis. These are studies with Wnt3a deficient mice [Luis et al., 2009], DKK1 transgenic mice [Fleming et al., 2008] and mice with specific deletion of β-catenin using VAV-Cre [Zhao et al., 2007]. Blocking Wnt signaling by overexpression of DKK1 in the stem cell niche [Fleming et al., 2008], or deletion of Wnt3a renders HSCs incapable of self-renewal [Luis et al., 2009]. Importantly, under both these conditions canonical Wnt signaling is severely diminished, as demonstrated by Wnt luciferase reporter assays or Wnt–LacZ reporter mice (T.C. Luis and F.J.T. Staal, submitted).

Wnt3a deficiency results in early embryonic lethality around embryonic day 12.5 (E12.5). Wnt3a null embryos reduced numbers of HSCs were found in situ which were functionally impaired in their long-term repopulation capacity as observed in serial, competitive transplantations, while multilineage differentiation capacity was in general not affected [Luis et al., 2009]. This phenotypic reduction in HSCs numbers was also reflected in functional assays, that is, in non-competitive transplantation assays in which Wnt3a$^{-/-}$, in comparison to equal numbers of wild-type total fetal liver cells showed almost no capacity to repopulate sub-lethally irradiated mice.

Since other WNT proteins are expressed in fetal liver, Wnt3a animals were crossed with the Bat–Gal Wnt reporter mice [Maretto et al., 2003] to investigate the impact of absence of Wnt3a for the activation of the canonical Wnt signaling pathway in HSCs. In comparison to littermate Wt embryos in which approximately 10% of the cells within the LSK population showed active Wnt signaling, the activation of this pathway was completely abolished in Wnt3a$^{-/-}$ fetal liver LSKs (T.C. Luis and F.J.T. Staal, submitted). Thus, WNT3a has a non-redundant role in the regulation of the HSC compartment and is the sole Wnt protein responsible for inducing canonical Wnt signaling in FL HSC.

A similar phenotype was also observed by Scadden and coworkers when HSCs transiently occupied a niche that over-expresses the naturally occurring canonical Wnt signaling inhibitor Dkk1 using an osteoblast-specific promoter to express Dkk1. Wnt signaling was markedly inhibited in HSCs which resulted in a progressive decline in hematopoietic reconstitution after transplantation [Fleming et al., 2008]. These HSC also lost competitive abilities in secondary transplantations. In the third study, Reya and coworkers mainly focused on interactions of β-catenin with BCR-ABL in the context of the development of a CML-like disease. However, they also showed that normal HSC, after VAV-Cre mediated deletion, performed poorly in competitive reconstitution experiments. Together, these studies demonstrate that HSC require canonical Wnt signals to maintain stem cell properties.

An intriguing fact, noticed by both our laboratory and that of David Scadden, is that this phenotype is irreversible, that is, transplantation into wild-type (Wnt competent) hosts does not restore the self-renewal defect. Thus loss of canonical Wnt signaling led to an irreversible impairment of stem cell function. This interesting phenomenon can possibly be explained by epigenetic modifications as a result of the absence of Wnt activation in HSC.

Taken together, these studies suggest that overexpression of Wnt signaling components can potentially enhance HSC self-renewal and in vivo reconstitution of irradiated recipients, however the exact dosage is critical and currently the optimal Wnt dosage for HSC function remains to be determined. However, loss of canonical Wnt signaling leads to impairment of HSC self-renewal.

LOSS-OF FUNCTION STUDIES USING Mx-Cre AND FLOXED β-CATENIN

In contrast to the above-described loss of Wnt signaling studies, no phenotype was observed in other studies using mice with deletion of β-catenin and its related homologue γ-catenin (also known as plakoglobin). Radtke and coworkers used HSCs that had a germline deletion for γ-catenin and were deleted for β-catenin using Mx-Cre. Completely unexpectedly, hematopoiesis and lymphopoiesis proceeded normally, leading the authors to exclude a role for Wnt signaling in both hematopoiesis and lymphopoiesis [Koch et al., 2007]. A similar approach to delete both β- and γ-catenin was also taken by Held and coworkers with similar results [Jeannet et al., 2007]. However, using reporter assays that had previously been used

to demonstrate TCF/LEF transcriptional activity [Reya et al., 2003; Weerkamp et al., 2006a], Held and coworkers documented that the Wnt-signaling pathway remained active when both β- and γ-catenin were deleted which have no overt impairment in hematopoiesis or lymphopoiesis [Cobas et al., 2004; Jeannet et al., 2007; Koch et al., 2008]. Despite the loss of both these known mediators, canonical Wnt signaling remained intact in their system leading them to invoke the exciting possibility of an additional β-catenin homolog in lymphocytes that may compensate for the loss of both β- and γ-catenin. It also should be noted that the disparate results from these Mx-Cre experiments may have something to do with unknown side effects of the Mx system, as the same floxed β-catenin mice crossed to VAV-Cre mice showed HSC defects [Zhao et al., 2007] and when crossed to Lck-Cre mice showed marked defects in thymocyte development [Xu et al., 2003]. It is currently unclear why this is, but perhaps the effects of interferons on very dormant populations of HSC [Essers et al., 2009] may mask some of the otherwise discernable effects. Additional studies will prove worthwhile in understanding this issue. Finally, on a philosophical note, the Wnt signaling pathway is strictly regulated and when deregulated, leads to malignant transformation [Weerkamp et al., 2006b; Guo et al., 2007], suggesting it is unlikely that this pathway would be active but non-functional in any tissue. Collectively these studies show that not all aspects of Wnt signaling in hematopoiesis are currently fully established and that further investigation is required to understand the role of canonical Wnt signaling in hematopoiesis and lymphopoiesis.

Wnt SIGNALING IN THE THYMUS

It is useful to contrast the role of Wnt signaling in HSC with its role in the thymus. Actually, the first reports of the importance of Wnt signaling in blood cells arose from studies on Wnt signaling in the context of T cell development in the thymus. Using soluble Frizzled receptors as scavengers for Wnt proteins, thereby inhibiting Wnt signaling, it was shown that such receptors inhibit T cell development, apparently largely by effecting thymocyte proliferation [Staal et al., 2001]. In accordance with this, thymi of Wnt1 × Wnt4 double deficient mice showed lower cellularity [Mulroy et al., 2002]. Recent work in $Wnt3a^{-/-}$ fetal thymi also showed a strong reduction in cell numbers and inhibition of development at the proliferative ISP and DN stages [Luis et al., 2009], reminiscent of the thymi from Tcf1 deficient mice [Verbeek et al., 1995].

Overexpression of inhibitory or dominant negative molecules in the Wnt pathway also have shown an important role for Wnt signaling in thymic T cell development. Expression of the cell autonomous inhibitor of β-catenin and Tcf (ICAT) [Pongracz et al., 2006], which inhibits Wnt signaling by preventing binding β-catenin to Tcf/Lef, blocks the earliest stages of T cell development in the thymus. Similarly, the secreted Wnt inhibitor DKK1, which blocks binding of Wnt to the required LRP coreceptor, inhibits thymocytye differentiation at the most immature stages. Activation of the pathway, by overexpressing activated forms of β-catenin led to generation of more thymocytes [Mulroy et al., 2003], allowed bypassing pre-TCR signals in mice lacking a pre-TCR [Gounari et al., 2001] and activated proliferation associated genes in immature thymocytes [Staal et al., 2004]. Importantly, Misra Sen and coworkers showed that conditional T cell specific deletion of β-catenin using the proximal Lck promoter to control Cre expression impaired T cell development at the β-selection checkpoint, leading to a substantial decrease in peripheral T cells [Xu et al., 2003].

Collectively, these studies have demonstrated a crucial role of Wnt signaling in the most immature stages of T cell development, where Wnt signals provide crucial proliferative factors. Given that only a few cells seed the thymus, an organ with billions of cells, massive proliferation is an essential feature of early thymocyte development.

INTERACTIONS WITH OTHER PATHWAYS

The consequence of Wnt signaling, stabilization of β-catenin results in cross-talk between Wnt, Sonic Hedgehog and Notch pathways. The final outcome is dependent on different synergistic and antagonistic environmental signals that form a complex network. The Notch signaling pathway was proposed to collaborate with Wnt signaling to maintain stem cells properties [Duncan et al., 2005]. In our studies with $Wnt3a^{-/-}$ HSC, no differences in Notch activity were observed. Therefore, it is unlikely that differential Notch signaling explains the lack of self-renewal of Wnt3a-defcient HSCs. However, this result does not exclude that Notch signals may still contribute to regulation of self-renewal, although recent reports have not shown evidence for such a role.

Elegant recent work, starting in zebrafish and extrapolated to mice, have found an unexpected link between PGE2 and Wnt signaling [Goessling et al., 2009]. The WNT and PGE2 signaling pathways were shown to be connected with stem cell activity in the hematopoietic system. In a recent issue of cell, these investigators use the zebrafish model to reveal a conserved PKA-dependent mechanism that connects the two pathways via β-catenin, enhancing stem cell proliferation and tissue regeneration [Goessling et al., 2009]. This mechanism not only operates in HSC but also in other types of stem cells.

Connections between Wnt and BMP signaling also have been proposed, as well as between Wnt and Hedgehog signaling. It seems likely that a number of conserved pathways operate in the HSC niche, as has been elegantly reviewed in a number of recent publications [Wilson and Trumpp, 2006; Blank et al., 2008].

SUMMARY AND FUTURE PERSPECTIVES

In contrast to the thymus, Wnt signals provide cell fate determining signals to HSC in the stem cell niche (Fig. 2). The majority of current evidence suggests that Wnt signaling is intimately involved with maintenance of stem cell properties such as self-renewal. The question remains as to whether in all cases, self-renewal of HSCs is solely mediated through β-catenin or can be regulated via other mediators. It will be especially important to develop in vivo model systems that allow manipulation of the dose of WNT signaling that cells receive, not only to solve some of the current controversies

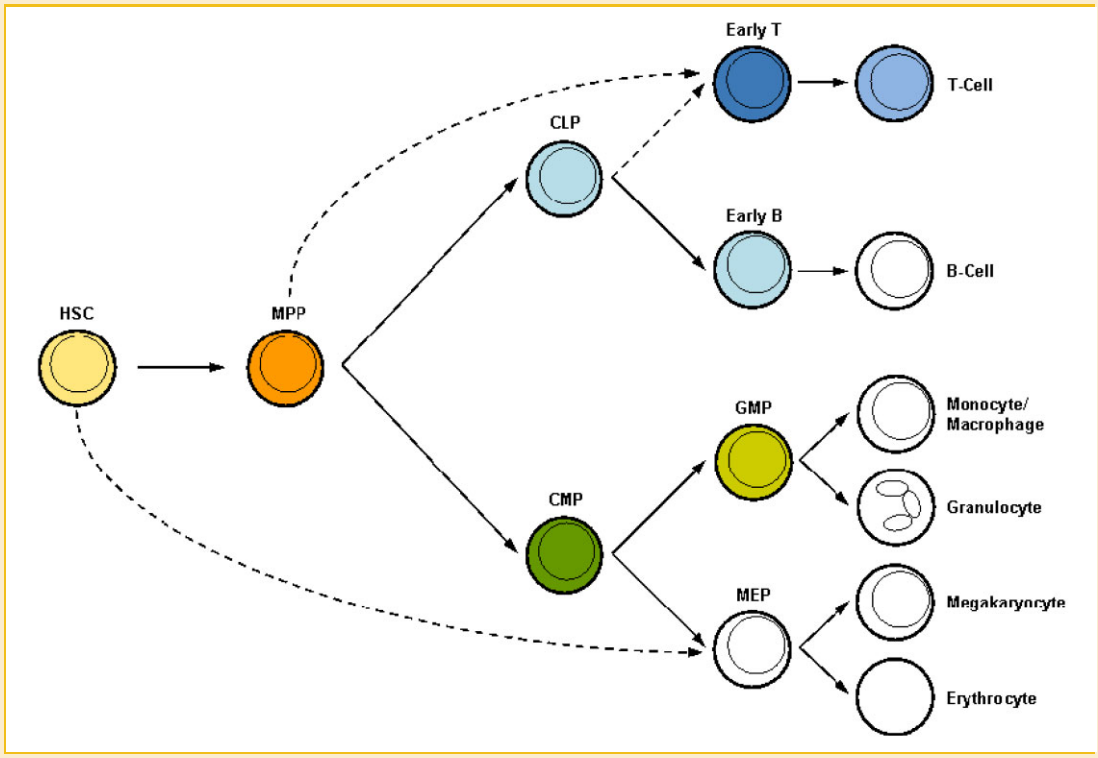

Fig. 2. Differential requirements of Wnt signaling during hematopoiesis. The darker the color, the more active Wnt signaling is. In uncolored lineages or cell types, Wnt signaling plays no reported role in normal development, but might be involved in leukemogenesis.

around optimal Wnt dosage, but also to translate these findings into therapeutic manipulations of the WNT pathway for clinical applications such as HSC expansion and thymic reconstitution.

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