ORIGINAL PAPER

Hedgehog signalling in vascular development

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Abstract The Hedgehog family of proteins are powerful
morphogens mediating embryonic development as well as
adult morphogenesis and carcinogenesis. For example,
excess hedgehog activity has been implicated in basal cell
carcinoma, medulloblastoma and rhabdomyosarcoma.
More recently, hedgehog signalling has been implicated in
angiogenesis. While hedgehog signalling in adult angio-
genesis may constitute a simple recapitulation of that in
embryonic development, it should be appreciated that
Hedgehog signalling occurs in embryonic angiogenesis in
different developmental contexts. This article reviews the
role of Hedgehog signalling in both embryonic and post-
natal vascular development. The temporal importance of a
window of hedgehog dependent angiogenesis during
development is emphasised and illustrated using a whole
mouse embryo culture system.

Keywords Angiogenesis · Hedgehog signalling ·
Indian hedgehog · Sonic Hedgehog · Motor neuron ·
Whole embryo culture · Yolk sac

Introduction

The Hedgehog's (Hh's) are a family of morphogenic pro-
teins mediating the development of various organs such as

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the nervous system, face, limbs and skin amongst others
[1]. Hh signalling serves a pivotal role in postnatal mor-
phogenesis such as tissue homeostasis, repair of damaged
tissue and tumorigenesis. Such roles can often be inter-
preted as a recapitulation of the Hh functions in embryonic
processes, as seen with other major signalling molecules in
development such as the fibroblast growth factors (FGFs),
transforming growth factors β's (TGFβ's) and Wnt family
members. We previously designated these molecules as
"stem molecules", because they are active in develop-
mental processes such as induction of epithelial
mesenchymal interactions and are utilised repeatedly in
ontogeny and phylogeny [2].

Since the seminal paper by Pola et al. in 2001 [3], Hh
signalling has attracted more attention in its role in angi-
ogenesis. Recent studies have shown that sonic hedgehog
(Shh) and Indian Hedgehog (Ihh) mediate embryonic vas-
cular development in the yolk sac, aorta and coronary
vessels [4, 5]. It is also known that postnatal or adult
angiogenesis can be enhanced by Hh signalling. As a
result, Hh signalling has become a target for therapeutic
angiogenesis in preclinical animal studies of ischemic
limbs, myocardial infarction and wound healing [5, 6].

There are several features worthy of consideration in Hh
signalling in angiogenesis. First, the physiological func-
tions of Hh signalling are broader than other more
angiogenesis-specific factors, such as vascular endothelial
growth factors (VEGFs) or angiopoietins (Angs). Second,
their role in embryogenesis is temporo-spatially deter-
mined by the developmental stage and context [2]. This
means that there is greater chance for Hh signalling to act
as a coordinator of crosstalk between angiogenesis and
other developmental processes such as neurogenesis. On
the other hand, since Hh signalling is so multi-functional,
there is a concern that therapeutic anti-angiogenic targeting

of Hh signalling molecules may be accompanied by unexpected angiogenic or other morphogenetic effects. Detailed investigation of the time-dependent actions of Hh induced angiogenesis is essential before clinical applications can be considered.

In this article, we review Hh signalling cascades and several examples of Hh dependent vascular development in embryonic and postnatal tissue. We also describe time windows of Hh dependent angiogenesis in the murine embryo that is illustrated using a whole embryo culture system.

Hh Signalling in vascular development

Hh signalling pathways

Figure 1 gives a brief summary of the hedgehog signalling cascade which is discussed in detail elsewhere [2, 7]. There are three vertebrate homologues of Drosophila Hh, Shh, Ihh and Dhh (desert hedgehog). Following modification with cholesterol and palmitate [8], Hh ligands are released from the producing cells with the help of the 12-trans-membrane protein Dispatched [9]. Hh ligands form a morphogen gradient and the signal is transduced by the
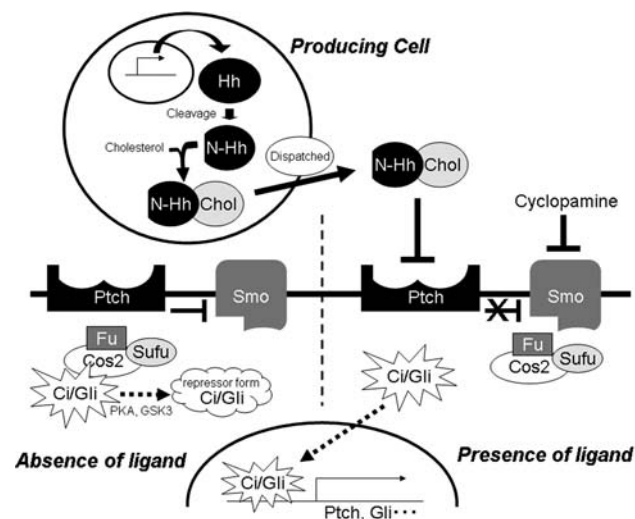

Fig. 1 *Outline of the hedgehog signalling pathway.* The hedgehog (Hh) ligand undergoes post-translational modification and then released from the cell through the membrane protein Dispatched. When the Hh ligand is absent, a receptor Patched (Ptch) inhibits the co-receptor Smoothend (Smo) and Costal-2 (Cos2)/Fused (Fu)/ Suppressor or fused (Sufu) complex converts a transcription factor Cubitus interruptus (Ci: Gli is the vertebrate homologue) into a repressor. When the Hh ligand is present, Hh binds to and represses Ptch, thereby releasing the activity of Smo. Smo recruits the Cos2 complex to the cytoplasmic membrane, and active-form Ci/Gli becomes available as a transcription factor. Reprinted from Nagase-T and Nagase-M [2] with permission from the publisher, NOVA Science

receptor Patched (Ptch) and co-receptor Smoothened (Smo) [1, 7]. Hh binding inhibits Ptch functions, but as the activity of the co-receptor Smo is inhibited by Ptch, Smo and its downstream transcription factor Gli are eventually activated by Hh ligands. The mechanisms of Gli regulation by Ptch/Smo interaction have been investigated mostly in *Drosophila*, in which Cubitus interruptus (Ci) is a homologue of Gli [10]. When Hh ligands are absent, Ci becomes a repressor [11] by the help of a complex containing Ci, Costal2 (Cos2) and the fused (Fu) serine/threonine kinase. When Smo is activated by Hh ligands, Smo can interfere with the Cos2 complex and active Ci accumulates in the cytoplasm and functions as a transcription factor [12]. The steroidal alkaloids cyclopamine (11-deoxyjervine) and jervine are natural antagonists of Hh signalling by influencing the balance between the active and inactive forms of the smoothened protein [13].

Shh is the most studied member of the Hh family. It regulates the development of the limb bud and central nervous system, for example, the induction of the motor neurons [14–16]. Gross anomalies of the central nervous system, including cyclopia (one eye) are observed in the Shh null mice [17]. Shh signalling is repeatedly used in later development, such as in the development of skin appendages [18]. Shh signalling is also involved in postnatal tumorigenesis, notably in skin and brain cancer [18, 19]. Ihh mediates endochondral osteogenesis and vascular development of the yolk sac (which is discussed later in detail) and Dhh mediates Schwann cell development. Finally, we note that the molecular cascade of Hh signalling is characterised by three properties [7] (i) long range diffusion of the ligands, (ii) positive and negative feedback loops within the cascade and (iii) biophysical or biomechanical modulation of the signalling.

Hh signalling in embryonic vascular development

A role for Shh in normal embryonic angiogenesis was first reported by Lawson et al. [20]. It was shown that Shh signalling derived from the notochord stimulates the formation of the aorta in the zebrafish embryo. Vokes et al. [21] described essential roles for Shh signalling in dorsal aorta formation in avian and mouse embryos. Shh is expressed in the avian endoderm and formation of the dorsal aorta was significantly inhibited in the endoderm-less or cyclopamine treated avian embryo as well as in Smo deficient mouse embryos at embryonic day (E) 7.5–8.0 (four to eight somite stage).

In addition, Shh signalling stimulates angiogenesis in various embryonic tissues at later developmental stages. For example, Lavine et al. [5, 22] showed that Shh is released from the epicardial layer of the heart in E11.5– 13.5 mouse embryos. Shh expression is triggered by FGFs

and it upregulates the expression of VEGFs and Ang-2 in the myocardium. The latter one then promotes the development of coronary vessels in the subepicardial space and within the myocardium. The developing lung vasculature is another target of Shh signalling, as pulmonary airway branching is defective in Shh null mice embryos. For example, Van Tuyl et al. [23] reported that the expression of Ang-1 and its receptor Tie-2 are downregulated in the lungs of E11.5–12.5 Shh null embryos and they speculated that defective angiogenesis results in impaired airway branching. In contrast, White et al. [24] reported that FGF9 and Shh synergistically regulate pulmonary capillary network formation via mesenchymal expression of VEGF and not Ang-1.

Several in vitro studies showed that Shh mediated migration and capillary formation of mature endothelial cells and putative endothelial progenitor cells from the bone marrow [25–27]. These studies confirmed that endothelial cells are a direct target of Shh. On the other hand, many reports have also shown indirect actions of Shh mediated angiogenesis [3], as seen in the aforementioned examples of coronary and pulmonary angiogenesis. Shh dependent aorta formation in zebrafish, reported by Lawson et al. [20], is also mediated by expression of VEGF in the mesoderm. Shh treatment in vitro upregulates angiogenic genes, such as VEGF and angiopoietins in NIH3T3 embryonic fibroblasts as well as in adult dermal fibroblasts [3, 28]. Altogether, these findings support a dual mechanism of Shh induced angiogenesis, namely, direct and indirect.

Ihh has been identified as a key regulator of murine yolk sac angiogenesis [4]. A primitive vascular network in the E8–8.5 mouse yolk sac develops into a mature vascular plexus penetrated by thick vessels within 24 h. The developing yolk sac provides a good experimental model, in which to study the transition from vasculogenesis to angiogenesis. Byrd et al. [29] demonstrated that the vasculature of Ihh−/− or Smo−/− murine yolk sacs failed to develop beyond a primitive vascular plexus and did not progress up to the angiogenic stage of development. The role of Ihh in yolk sac vasculogenesis and angiogenesis is discussed later in detail. Ihh signalling also serves a pivotal role during skeletogenesis in the extremities. Ihh is expressed in the hypertrophic chondrocytes of developing endochondral bones and Ihh null mice show defective osteogenic differentiation [30]. Further, Colnot et al. [31] reported that the penetration of blood vessels into the ossification site is impaired in Ihh deficient mouse embryos. This vascular phenotype appears autonomous of the endothelial cells and it was not mediated by environmental angiogenic factors, since co-grafting of the Ihh null vessels with wild type surrounding tissues gave rise to the same vascular defects [31].

Hedgehog signalling in postnatal vascular development

The role of Shh signalling in postnatal and adult angiogenesis is also of a great interest, especially as a potential pharmacological target. Pola et al. [3] demonstrated that administration of Shh protein induces angiogenesis in ischemic limbs and the cornea of adult mice. Kusano et al. [32] reported induction of nerve vessels and restoration of nerve function in rat diabetic neuropathy after systemic injection of Shh protein, suggesting a therapeutic angiogenic potential of exogenous Shh. Nerve regrowth then followed angiogenesis. In these cases, VEGF expression was upregulated by Shh signalling in the corneal stromal cells and "nerve fibroblasts", respectively [3, 32].

It is of interest to consider whether endogenous Shh signalling in postnatal vascular development is a physiological and/or repair process. Shh expression was upregulated in regenerating muscle in mouse ischemic legs [33]. Thus, administration of an Shh-neutralising antibody inhibited angiogenesis and VEGF upregulation in this model, implying a role for endogenous Shh signalling in ischemic angiogenesis. Similar findings were reported by Surace et al. [34], who showed that retinal angiogenesis due to ischemia was associated with upregulation of Shh signalling and VEGF, and that these effects were antagonised by cyclopamine. Asai et al. [27] reported that endogenous Shh signalling is activated in a mouse wound model and the transfection of naked Shh DNA promoted wound healing via vasculogenesis, i.e. recruitment of the so-called endothelial progenitor cells from the bone marrow. However, this paper failed to show clear evidence for involvement of activated endogenous Shh signalling in the wound healing and there was no use of signal pathway inhibitors. Nevertheless, these data point to the involvement of Shh signalling in angiogenesis and vasculogenesis in response to tissue injury.

An area of recent interest is the potential of modulating Shh signalling as a therapy for myocardial ischemia. Conditional activation of Gli-2 in the mouse adult heart, resulted in increased coronary vessel density within the myocardium along with upregulation of VEGF [22]. Furthermore, Shh gene therapy restored myocardial function in a mouse model of acute or chronic myocardial infarction [35]. In this model, transfection of DNA encoding human Shh not only promoted neovascularization via, at least in part, recruitment of endothelial progenitor cells, but also reduced cardiac fibrosis and apoptosis. Several angiogenic factors, such as VEGF and angiopoietins, were upregulated by Shh in the cardiac fibroblasts [35]. Altogether, these data suggest that Shh signalling during ischemia results in upregulation of VEGF, thereby promoting angiogenesis.

Finally, Shh signalling has been implicated in the development of several malignancies including basal cell carcinoma of the skin, lung cancer and medulloblastoma

[18, 19], and it is possible that Shh mediates tumour angiogenesis [36]. The expression of Hedgehog-interacting protein, a natural antagonist of Hh ligands highly expressed in normal vascular endothelial cells, is downregulated in several human cancers. As a result, Hh signalling may be enhanced, stimulating tumour angiogenesis [37].

Windows of Hh dependent angiogenesis in mouse embryos

The Shh knockout mouse shows a gross cyclopic phenotype [17]. To further probe Shh function in embryogenesis, a series of studies using a whole embryo culture system were carried out, concentrating on 'time windows' of hedgehog dependent angiogenesis [2, 38–41]. The sterodial alkaloids cyclopamine and jervine specifically target Smo, thereby inhibiting Hh signalling [13] and were used as functional probes. Technical details of the whole embryo culture system have been described previously [41, 42].

Vascular defects in the yolk sac and aorta of cyclopamine treated mouse embryos E7.8–9.5

Mouse yolk sacs are a good model for analysing vasculogenesis and angiogenesis, since the primitive vascular network arising from vasculogenesis at around E8.5 can be clearly distinguished from the more extensive network arising from angiogenesis at E9.5. lhh−/− or Smo−/− murine yolk sacs show the primitive vascular plexus but fail to undergo angiogenic remodelling [29]. The impaired angiogenesis observed in the knockout mice could be attributable to a true defect in angiogenesis at this stage or may be a sequel of earlier vasculogenic failure, since lhh signalling has also been reported to be required for the development of blood islands and endothelial tube formation earlier in mouse embryogenesis [21, 43]. This prompted studies to examine the role of Hh by conditionally blocking hedgehog signalling within a narrow time frame when the earlier vasculogenesis is already complete [40].

Figure 2 compares the morphology of the yolk sac vasculature in control, 20 μM cyclopamine and 20 μM jervine treated embryos at E8.5 and E9.5. The E8.5 yolk sac shows immature vascular networks generated by vasculogenesis. At E9.5, the primitive plexus in the control group has been remodelled into a ramified structure with distinct large and small vessels. In contrast, the yolk sac vessels in the cyclopamine and jervine groups show immature vasculogenesis even at E9.5 [40]. Northern blotting and in situ hybridization showed expression of lhh, but neither Shh nor Dhh in the yolk sac. These results

Fig. 2 *Defective yolk sac angiogenesis after Hh signalling blockade during E7.8–9.5* (**a–f**) Whole-mount PECAM-1 immunostaining of cultured yolk sacs from the control group (Cont; **a**, **d**), the 20 μM cyclopamine treated group (Cy20; **b**, **e**) and the 20 μM jervine treated group (Jer20; **c**, **f**) harvested at E8.5 (**a–c**) and E9.5 (**d–f**). (**g**, **h**) Whole-mount Flk-1 immunohistochemistry of cultured yolk sacs from Cont (**g**) and Cy20 (**h**) groups harvested at E9.5. Yolk sacs were mounted two dimensionally on a glass slide. The ramified angiogenesis is observed in the E9.5 Cont groups (**d**, **g**), whereas a primitive capillary network is seen in the E9.5 Cy20 or Jer20 groups (**e**, **f**, **h**). Scale Bar, 100 μm. Reprinted from Nagase-M et al. [39] with permission from Elsevier
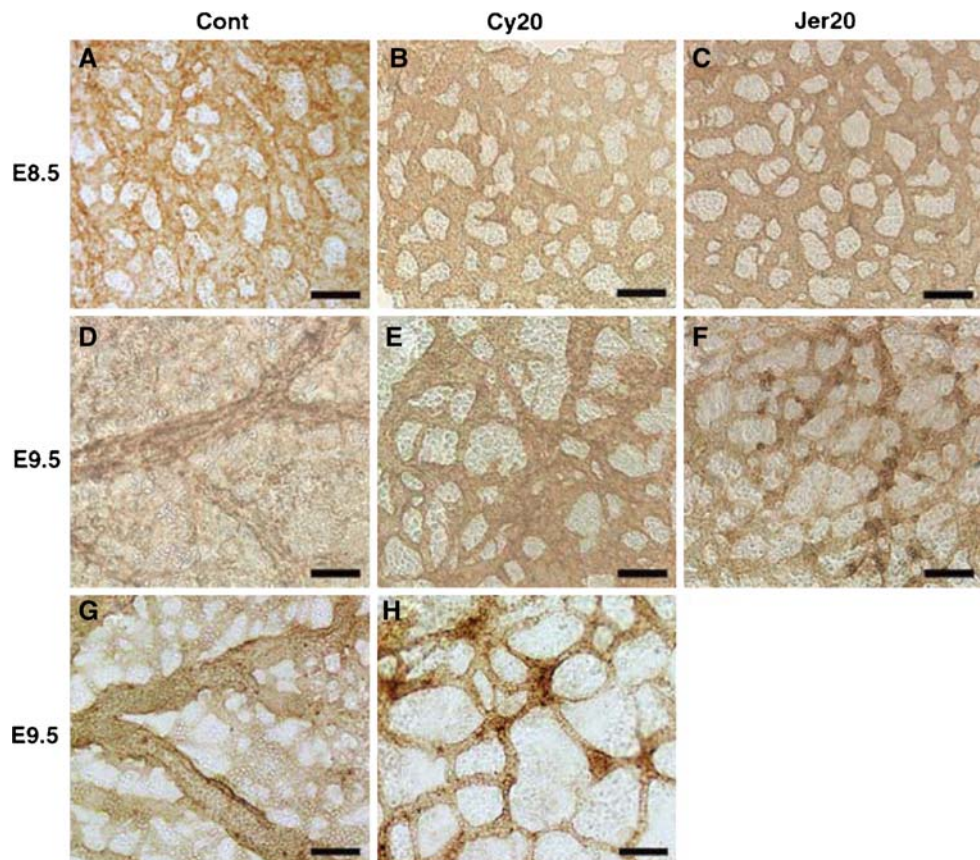
suggest that lhh signalling mediates the transition from vasculogenesis to angiogenesis. This is in addition to the previously recognised role of Ihh in blood island formation and vasculogenesis of the yolk sac at an earlier developmental stage [29, 43]. VEGF, Flk-1 and Notch-1 were downregulated in the yolk sacs of the cyclopamine treated group, but there were no expression changes in Delta-like 4 (a ligand for Notch-1), Ang-1,2 or Tie-2, suggesting VEGF and Notch are involved downstream of lhh signalling.

Cyclopamine also impairs fusion of the two dorsal aortae in embryos during E7.8–9.5 [39]. From E7.8 to E8.5, a pair of dorsal aortae could be clearly recognised in both the control and cyclopamine treated groups. However, by E9.5, the aorta was normally fused at the limb bud level in the control group, whereas fusion did not occur in the cyclopamine treated embryos. Shh was expressed in the notochord and the floor plate in close vicinity to the fused aorta, implicating Shh as a mediator of aortic fusion. Expression of BMP4 and VEGF, putative downstream genes of Shh, were downregulated around the aorta following cyclopamine treatment [39].

Vascular defects in the craniofacial regions and the neural tube of cyclopamine treated embryos at E8.5 and E10.5.

Vascular defects caused by cyclopamine administration during E8.5–10.5 are quite different from those following administration during E7.8–9.5, suggesting time-specific angiogenic effects of Hh signalling. The embryos treated with 20 μM cyclopamine from E8.5 to E10.5 exhibited a craniofacial anomaly resembling mild holoprosencephaly [42]. This morphological change was associated with dilation of the craniofacial surface vessels, possibly the external cardinal veins [39]. Whole mount PECAM-1 immunohistochemistry revealed that the vascular network in the craniofacial region of the cyclopamine embryos was coarser than that in the control embryos.

Defects in neural tube angiogenesis following cylopamine treatment in the same window were also reported [39]. Sprouting vessels from the perineural vascular plexus (PNVP) enter the neural tube at around E9.8–10.2 in normal embryos [44]. In cyclopamine treated embryos, the neural tube rounded and the arch-like sprouting that occurs from the PNVP was inhibited [38] (Fig. 3). A similar inhibition of vascular invasion into the neural tube was also reported in the Tie-2 knockout mouse embryo [45], suggesting the involvement of Ang-1/Tie-2 signalling in this process. Indeed, Ang-1 was expressed in the developing motor neurons towards which the vessel invasion occurs [38], suggesting that Ang-1 is a molecular cue for vascular sprouting. How does Hh signalling mediate these processes? Shh expressed in the notochord and the floor plate
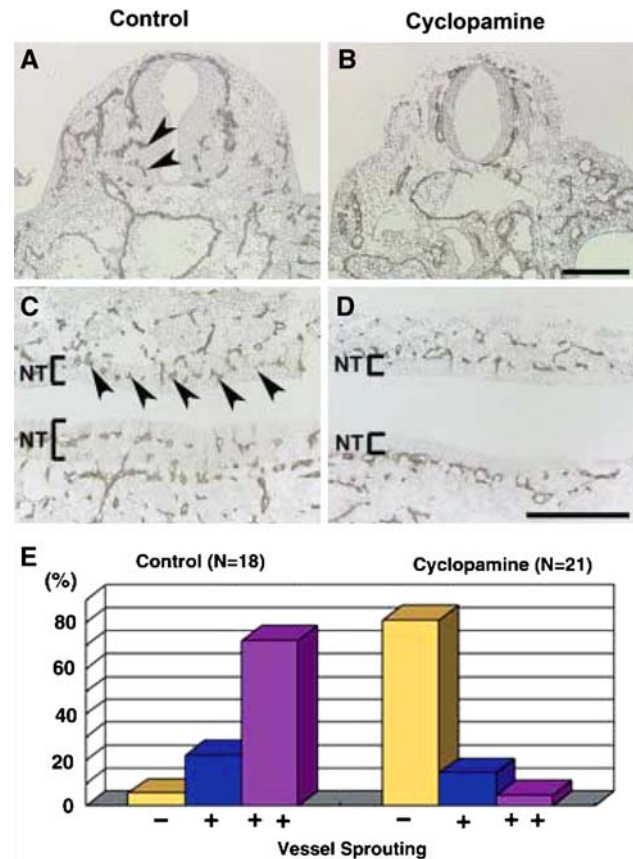

Fig. 3 *Defective angiogenesis within the neural tube after Hh signalling blockade during E8.5–10.5* (**a–d**) PECAM-1 frozen section immunostaining of the neural tube in the control group (**a**, **c**) and the cyclopamine treated group (**b**, **d**). The arch-like sprouting vessels are observed in the control embryos (arrowheads in **a**, **c**), which are missing on cyclopamine treatment (**b**, **d**). Scale Bar, 200 μM. (**e**) Percentages of the cultured embryos with different degrees of the vessel sprouting. Sprouting is categorised as follows:- Little sprouting. + Some sprouting is observed but the vascular arches are not yet formed. ++ Sprouting is more advanced and the vascular arches are nearly completed. Reprinted from Nagase-T et al. [38] with permission from Blackwell

plays a pivotal role in induction of the ventral motor neurons [14, 15]. The sprouting defects seen in the cyclopamine treated embryos may be due to impaired induction of the motor neurons, since the Ang-1 positive motor neurons were largely missing in the cyclopamine treated embryo [38]. This finding is a novel, unexpected example of Shh dependent angiogenesis via induction of the motor neurons, illustrating tight coordination of angiogenesis and neurogenesis.

Conclusions

It is now clear that members of the hedgehog family of morphogenesis are actively involved in angiogenesis at

various stages of mouse development. Several reports point to the involvement of VEGF and Notch-1, as well as angiopoietins, though further work is needed to fully elucidate what signalling mediates this angiogenesis.

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