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ORIGINAL ARTICLE

GATA4-Twist1 Signalling in Disturbed Flow-Induced Atherosclerosis

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Abstract

Background Endothelial cell (EC) dysfunction (enhanced inflammation, proliferation and permeability) is the initial trigger for atherosclerosis. Atherosclerosis shows preferential development near branches and bends exposed to disturbed blood flow. By contrast, sites that are exposed to non-disturbed blood flow are atheroprotected. Disturbed flow promotes atherosclerosis by promoting EC dysfunction. Blood flow controls EC function through transcriptional and post-transcriptional mechanisms that are incompletely understood.

Methods and Results We identified the developmental transcription factors Twist1 and GATA4 as being enriched in EC at disturbed flow, atheroprone regions of the porcine aorta in a microarray study. Further work using the porcine and murine aortae demonstrated that Twist1 and GATA4 expression was enhanced at the atheroprone, disturbed flow sites in vivo. Using controlled in vitro flow systems, the expression of Twist1 and GATA4 was enhanced under disturbed compared to non-disturbed flow in cultured cells. Disturbed flow promoted Twist1 expression through a GATA4-mediated transcriptional mechanism as revealed by a series of in vivo and in vitro studies. GATA4-Twist1 signalling promoted EC proliferation, inflammation, permeability and endothelial-to-mesenchymal transition (EndoMT) under disturbed flow, leading to atherosclerosis development, as shown in a combination of in vitro and in vivo studies using GATA4 and Twist1-specific siRNA and EC-specific GATA4 and Twist1 Knock out (KO) mice.

Conclusions We revealed that GATA4-Twist1-Snail signalling triggers EC dysfunction and atherosclerosis; this work could lead to the development of novel anti-atherosclerosis therapeutics.

Keywords Atherosclerosis · Endothelial cell dysfunction · Developmental signalling · EndoMT

Introduction

Atherosclerosis is a chronic, inflammatory disease of the vasculature that gives rise to heart attacks and stroke. Endothelial cells (EC) that line the vasculature play a central role in mediating vascular health, and EC dysfunction is the initial trigger for atherosclerosis. ECs are in direct contact with the flowing blood that exerts a mechanical drag known as shear stress. Despite the contribution of systemic risk factors (age, obesity, high cholesterol, age, smoking), atherosclerosis shows a non-random distribution in the vasculature. Atherosclerotic plaques show a preferential development at bends and branch points; these vascular sites are exposed to disturbed blood flow, which generates multidirectional, low wall shear stress, whereas sites that are exposed to non-disturbed flow, which exerts uniform, high shear stress, are atheroprotected [1–4]. Disturbed flow induces atherosclerosis development by promoting EC dysfunction (enhanced inflammation and proliferation) [3–6]. As a result of enhanced proliferation, ECs lose contact with neighbouring cells and become more permeable to inflammatory cells and molecules such as low density lipoproteins (LDL), leading to atherosclerotic lesion formation [7]. Blood flow promotes EC dysfunction through modulating endothelial gene expression by molecular mechanisms that are not fully understood [4, 8–11].

To further characterize the molecular mechanisms that control focal atherogenesis, we conducted a microarray study which revealed that the transcription factors Twist1 and GATA4 showed enhanced gene expression in EC at disturbed flow, atheroprone regions of the porcine aorta [12]. Both of these transcription factors control major developmental processes, Twist1 controls gastrulation during embryogenesis [13], whereas GATA4 is a master regulator of cardiac development [14]. Although the role of Twist1 and GATA4 in embryonic

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development is well characterized, their potential function in ECs and vascular disease is entirely unknown.

The Role of GATA4 and Twist1 in Embryonic Development

GATA4 Controls Heart Development

GATA4 is a zinc finger transcription factor that belongs to the GATA family, containing GATA1-GATA6 proteins. GATA proteins play non-redundant roles and display tissue-specific expression; GATA1–3 are more closely related in sequence and are predominantly expressed in the hematopoietic system, whereas GATA4–6 are more closely related and are expressed in the cardiovascular system and the gonads [15]. The process of mammalian heart development begins with looping of the early heart tube (consisting of cardiomyocytes and endothelial cells), which then becomes divided into four chambers that are separated by endocardial cushion-mediated valve formation. This process is controlled by a tightly regulated cardiac transcriptional network. GATA4 is regarded as a master cardiac transcription factor as it acts upstream of the transcriptional network controlling heart development, including myocyte enhancer factor (Mef)2c, NK2 homeobox 5 (Nkx2.5), serum response factor (Srf) and Hand2 [16–18]. Grepin et al. established the dominant role of GATA4 in cardiac development by revealing that GATA4 overexpression in pluripotent P19 embryonic carcinoma cells committed the cells to undergo differentiation to the cardiac lineage and gave rise to beating cardiomyocytes [19]. Genetic inhibition of GATA4 restricted beating cardiomyocyte differentiation [19], thus indicating that GATA4 expression is required to drive cardiomyocyte differentiation. Interestingly, when overexpressed in embryonic stem cells, GATA4 induces extraembryonic endoderm, not cardiomyocytes [20]. However, under defined culture conditions (serum-free media), GATA4 is able to efficiently promote cardiogenesis in embryonic stem cell derivatives [21]. Mice lacking GATA4 die before the onset of heart development due to defects in the extraembryonic endoderm [14, 22] while rescue experiments providing the null embryos with wild-type extraembryonic endoderm showed that GATA4 is required for cardiogenesis and proepicardium development [23].

Twist1 Plays a Major Role in Multiple Stages of Embryonic Development

Twist1 is a transcription factor that belongs to the basic Helix-Loop-Helix (bHLH) family of transcriptional regulators. Twist1 was originally discovered in Drosophila, as one of the genes essential for mesoderm specification [13]. Drosophila embryos lacking the Twist1 gene died during embryogenesis with a “twisted” appearance [13]. In mouse, Twist1 plays a crucial role during multiple stages of embryonic development, from controlling mesodermal differentiation during gastrulation (around embryonic day (E) 6) up to controlling valve formation at E 16 [24].

Twist1 also acts as a master regulator of the osteoblast differentiation programme by controlling the activity of downstream pathways that are central to osteoblast differentiation and development, including fibroblast growth factor (FGF) and bone morphogenic protein (BMP) signalling by controlling FGFR2, FGF8, FGF10, and BMP4 transcription [25–28].

GATA4 and Twist1 Promote Heart Valve Formation Through Endothelial-to-Mesenchymal Transition

GATA4 and Twist1 exert overlapping functions during atrioventricular valve development by inducing endothelial-to-mesenchymal transition (EndoMT), which describes a process in which mature endothelial cells adopt a mesenchymal phenotype. Markwald et al. discovered EndoMT as a critical process during heart valve development, where it was observed that cells of the endocardium acquire a more mesenchymal identity and participate to the formation of the mesenchymal heart cushion, the precursor of the cardiac valves [29]. Ultimately, EndoMT cells separate from the monolayer and penetrate the underlying extracellular matrix (a process referred to as delamination) and become highly migratory and invasive [30]. Therefore, enhancements in cell proliferation, permeability, migration, and invasion are all functional outputs of cells undergoing EndoMT. GATA4 and Twist1 are downstream targets (through direct transcriptional activation or through post-translational modifications) of EndoMT promoting pathways, i.e. TGFβ, BMP, Notch and Wnt signalling [31–35]. Interestingly, Twist1 and GATA4 can also act as positive regulators of these EndoMT promoting pathways [36–38]. In vivo studies have shown that endothelial-specific deletion of Twist1 or GATA4 blocks the EndoMT programme and subsequently heart valve formation [39]. Specifically, the EndoMT-promoting mechanism of Twist1 and GATA4 involves the transcriptional activation of mesenchymal genes Snail, Slug, α-Sma, and Cdh2 [39, 31, 40, 41].

Do GATA4 and Twist1 Play a Role in the Adult Vasculature and in Disturbed Flow-Induced Atherosclerosis?

In the disease context, GATA4 and Twist1 have been shown to drive cancer progression and survival by mediating metastasis and proliferation [42–45]. However, the role of GATA4 and Twist1 in the adult vasculature and in atherosclerosis is completely unknown. We recently demonstrated for the first time that disturbed flow promotes GATA4-dependent induction of Twist1 in EC. Studies using the zebrafish embryo revealed that Twist was expressed in early embryonic vasculature where it promoted angiogenic sprouting by inducing EC proliferation and migration. In adult mammalian arteries, GATA4 and

Twist1 were expressed preferentially at atheroprone sites exposed to disturbed flow where GATA4-Twist1 signalling promoted the development of atherosclerosis by inducing EC dysfunction (proliferation, permeability and inflammation) [46].

GATA4 and Twist1 Expression Is Enhanced by Atheroprone, Disturbed Flow

To investigate the expression of flow-mediated genes, we conducted a microarray study on ECs isolated from either disturbed blood flow sites (inner curvature) or non-disturbed flow (outer curvature) of the porcine aorta [12]. The study revealed that hundreds of genes showed differential expression under flow; this gene set included Twist1 and GATA4, which were identified as being enriched at the atheroprone, disturbed flow site. This observation was validated by qPCR studies of an independent cohort of pigs (Fig. 1 *A-C*). Similarly, en face staining of the murine aortic endothelium demonstrated elevated levels of Twist1 and GATA4 protein levels at the inner curvature of the aortic arch (disturbed flow) compared to the outer curvature (non-disturbed flow) (Fig. 1 *D-F*). These observations revealed that the expression of GATA4 and Twist1 is driven by atheroprone, disturbed flow in the vasculature.

To directly study the effects of disturbed flow on Twist1 and GATA4 expression, we used in vitro models of flow. Using two complementary systems, an orbital plate or a parallel plate system [47, 48], ECs were exposed to either low, oscillating shear stress (mimicking disturbed flow in vivo) or high, unidirectional shear stress (mimicking non-disturbed flow in vivo), and it was observed using both systems that low shear stress induced the expression of Twist1 and GATA4 mRNA and protein. To conclude, these data showed that disturbed flow drives the expression of GATA4 and Twist1 in vitro and in vivo [46].

GATA4 and Twist1 function as part of the same pathway during valve formation in embryonic development [30]; thus, we hypothesized that GATA4 and Twist1 may be acting in a pathway in response to disturbed flow in vascular ECs. We tested this hypothesis in vitro using a combination of gene silencing and chromatin immunoprecipitation experiments, which revealed that GATA4 acted upstream of Twist1 in ECs exposed to disturbed flow, where it promoted Twist1 expression through a transcriptional mechanism. To validate these observations in vivo, we assessed the effects of EC-specific genetic deletion of GATA4 ($Gata^{cKO}$) on endothelial expression of Twist1 in the murine aorta. En face staining

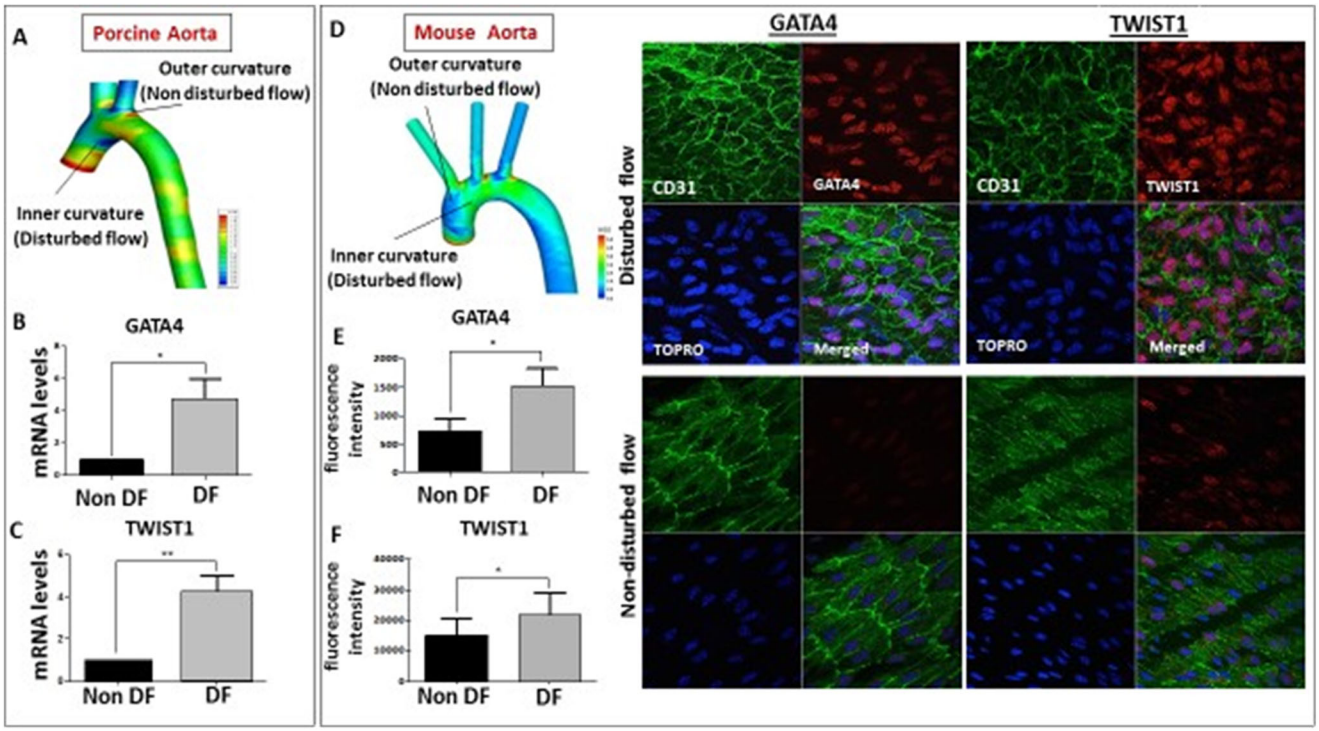

Fig. 1 GATA4 and TWIST1 were preferentially expressed at disturbed flow, atherosusceptible sites in vivo. (A–C) Expression of TWIST1 and GATA4 was assessed at disturbed flow sites (inner curvature of the aortic arch) and non-disturbed flow (outer curvature) regions of the porcine aorta by qRT-PCR analysis ($N = 5$). (D–F) Protein expression of GATA4 or TWIST1 in EC were assessed by en face staining of disturbed flow (inner curvature) or non-disturbed flow (outer curvature) regions of the murine aorta in C57BL/6 mice (red). EC were identified by co-staining with anti-CD31 antibodies (green). Cell nuclei were stained using TO-PRO-3 (blue). Representative images and quantitation of GATA4 or TWIST1 expression (mean ± SEM) are shown. Scale on the bottom right of figures A and D indicates wall shear stress magnitude (Pa), the red end of the spectrum indicates higher shear stress, and the blue indicates lower shear stress. $*p < 0.05$, $**p < 0.01$, using a paired t test. From Mahmoud et al. [46]

demonstrated that the expression of Twist1 at the disturbed flow area was reduced in Gata^{cKO} compared to control Gata4$^{flox/flox}$ mice, revealing that GATA4 positively regulates Twist1 in atheroprone, disturbed flow sites in vivo [46].

GATA4-Twist1 Signalling Promotes Atherosclerosis by Driving EC Dysfunction

Disturbed flow promotes EC dysfunction and subsequently atherosclerosis; since GATA4-Twist1 signalling was elevated in ECs at atheroprone, disturbed flow sites, we assessed whether GATA4-Twist1 signalling promoted disturbed flow-induced EC dysfunction (inflammation, proliferation, and permeability) and atherogenesis. This was tested using a combination of complementary approaches consisting of in vitro studies using cultured ECs and exposing them to flow using well-controlled systems (orbital plate and parallel plate systems) following gene silencing of Twist1 and GATA4 using siRNA, and in vivo studies using mice with an endothelial-specific deletion for GATA4 and Twist1. The results from these studies revealed that GATA4 and Twist1 promoted EC proliferation, permeability and inflammation under disturbed flow, reflecting that GATA4-Twist1 signalling promotes disturbed flow-induced EC dysfunction. The mechanisms by which GATA4-Twist1 signalling could be mediating EC proliferation involved the regulation of cell cycle genes such as Cyclins D1, G2, and CDK4 [46]. This observation is consistent with other studies that have demonstrated the role of GATA4 and Twist1 in driving proliferation and cell cycle regulation in other contexts [41, 49]. Another mechanism by which GATA4-Twist1 signalling drives EC dysfunction was through the induction of EndoMT [46, 50].

We next investigated whether GATA4-Twist1 signalling promoted atherosclerosis since it promoted EC dysfunction under disturbed flow. Using a PCSK9-adenoassociated virus followed by high fat diet feeding (6 weeks), we induced hypercholesterolemia in Gata4^{cKO} and Twist1^{cKO} mice (Fig. 2 *A*). Interestingly, atherosclerotic lesion formation was suppressed in mice lacking the GATA4 and Twist1 gene, compared to control mice, revealing that GATA4-Twist1 signalling mediated atherogenesis (Fig. 2 *B*). To conclude, GATA4-Twist1 signalling drives EC proliferation, permeability, inflammation (EC dysfunction) and atherogenesis in response to disturbed flow [46].

EndoMT and Atherosclerosis

Recent studies established the contribution of EndoMT to atherosclerosis [51–53]. EndoMT has notably been linked to plaque progression since the extent of EndoMT

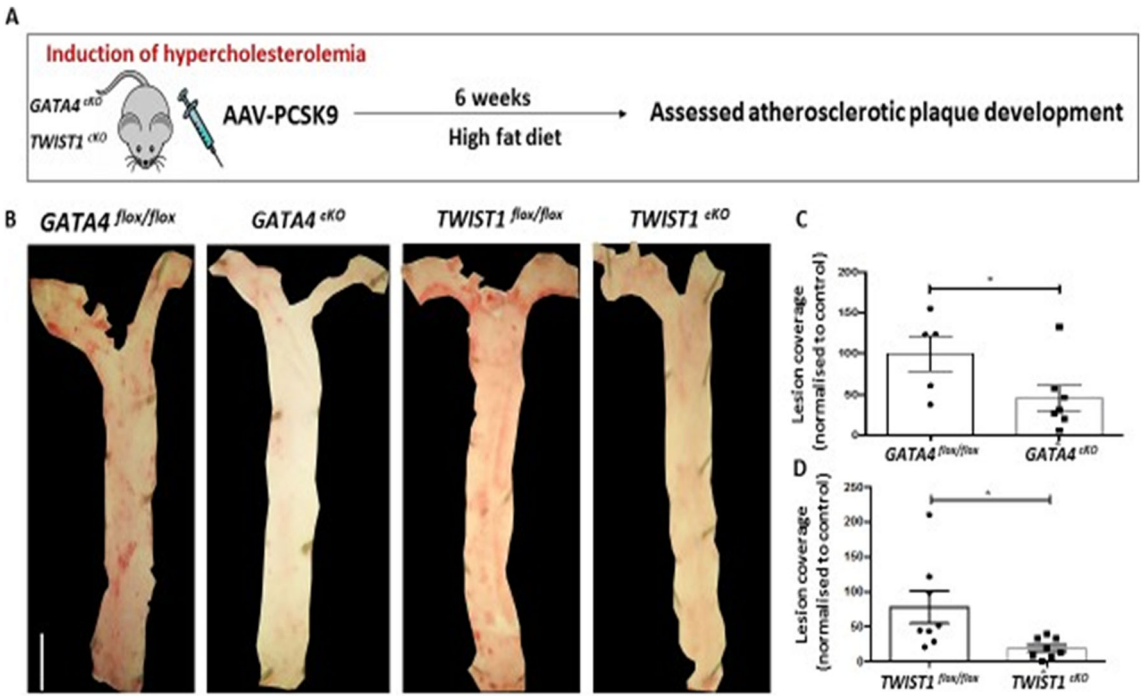

Fig. 2 GATA4 and TWIST1 promote atherosclerotic lesions at disturbed flow sites. **a** Experimental schematic, GATA4cKO or GATA4flox/flox mice and TWIST1cKO or TWIST1flox/flox mice were treated with AAV-PCSK9 to induce hypercholesterolemia. Following 1 week of AAV-PCSK9 injection, the mice were fed a high fat diet for 6 weeks. **b** Atherosclerosis lesions were stained using Oil Red O and quantified using image J software. Representative images are shown. Scale bar, 1 mm). **c**, **d** The percentage of atherosclerosis lesion coverage was calculated. Data were pooled from multiple mice and mean values ± SEM are shown. $^{**}p < 0.01$,$^{*}p < 0.05$ using an unpaired *t* test. From Mahmoud et al. [46]

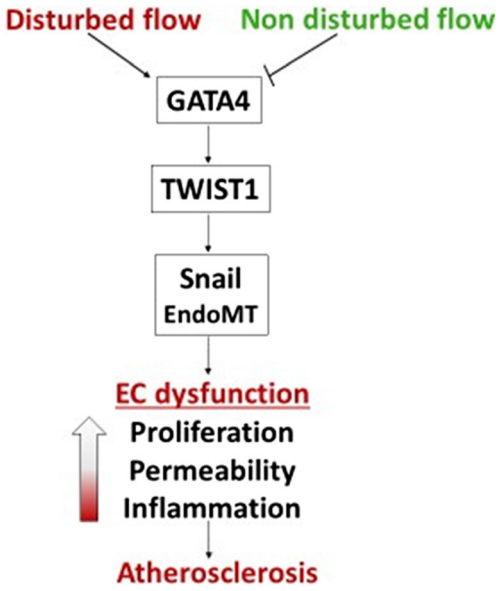

Fig. 3 Disturbed flow promotes EC dysfunction and atherosclerosis through activating GATA4-TWIST1-Snail signalling

observed in human plaques correlates strongly with the severity of the disease [52, 53]. Furthermore, endothelial lineage tracing studies in the mouse established the occurrence of EndoMT during plaque development [52, 53]. These elegant studies revealed that a large proportion of aortic EC delaminate and migrate into the atherosclerotic lesions and may promote plaque growth by increasing inflammation. EndoMT process has also been linked to disease initiation as Chen et al. demonstrated that inhibition of FGF, an antagonist of EndoMT, increases lesion initiation in a mouse model of atherosclerosis [52].

As Twist1 and GATA4 are regulators of EndoMT during embryonic development, we investigated whether the mechanism for GATA4-Twist1-driven EC dysfunction leading to atherosclerosis involves EndoMT. A series of studies revealed that disturbed flow induced EndoMT in cultured ECs and that GATA4 and Twist1 are positive regulators of this process. Further studies revealed that GATA4-Twist1 signalling directly activated the transcription factor Snail, a key driver of EndoMT, through a transcriptional mechanism [50]. In addition, we found that GATA4-Twist1-mediated activation of Snail contributed to proliferation and permeability in cultured ECs under disturbed flow [50]. Collectively, our observations reveal that GATA4-Twist1 signalling promotes EC dysfunction under disturbed flow via induction of EndoMT [46, 50]. Our study gives a new insight into the mechanisms controlling EC dysfunction in response to disturbed flow and, in accordance with Chen's studies, suggests that EndoMT plays a crucial role in the initiation of atherosclerosis [52].

Summary

The role of GATA4 and Twist1 in embryonic development is well established, whereas the role of GATA4 and Twist1 in adult arteries was previously unknown. Here, we reveal that the developmental GATA4-Twist1 signalling is regulated by mechanical forces in adult ECs, where atheroprone disturbed flow induced GATA4 expression in ECs, which in turn transcriptionally activated Twist1 expression. GATA4 and Twist1 have been shown to be regulated by mechanical forces in other contexts, GATA4 has been shown to be activated in cardiac cells in response to mechanical loading of the heart ventricles [54] and Twist1 to be regulated by mechanical forces exerted by tissue deformations during Drosophila embryonic development [55], and in response to matrix stiffness in tumour cells [56]. We show that disturbed flow-driven GATA4-Twist1 signalling promoted EC proliferation, permeability, and inflammation, key characteristics of dysfunctional ECs. As a result of inducing EC dysfunction, GATA4-Twist1 signalling promoted the development of atherosclerosis (Fig. 3). We propose that the mechanism by which GATA4-Twist1 signalling promoted EC dysfunction and atherosclerosis is in part mediated by the initiation of EndoMT, a process that has been recently linked with the progression of atherosclerosis [51–53]. These observations have implications for the development of new therapies to prevent the initiation or progression of atherosclerosis by targeting EndoMT.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

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