

**Homeobox A4 Suppresses Vascular Remodeling as a Novel Regulator of
YAP/TEAD Transcriptional Activity**

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Abstract

The Hippo signaling pathway is involved in the pathophysiology of various cardiovascular diseases. Yes-associated protein (YAP) and transcriptional enhancer activator domain (TEAD) transcriptional factors, the main transcriptional complex of the Hippo pathway, were recently identified as modulators of phenotypic switching of vascular smooth muscle cells (VSMCs). However, the intrinsic regulator of YAP/TEAD-mediated gene expressions involved in vascular pathophysiology remains to be elucidated. Here, we detected Homeobox A4 (HOXA4) as a potent repressor of YAP/TEAD transcriptional activity using lentiviral shRNA screen. Mechanistically, HOXA4 interacts with TEADs and attenuated YAP/TEAD-mediated transcription by competing with YAP for TEAD binding. We also clarified that the expression of *HOXA4* is relatively abundant in the vasculature, especially in VSMCs. *In vitro* experiments in human VSMCs showed HOXA4 maintains the differentiation state of VSMCs via inhibition of YAP/TEAD-induced phenotypic switching. We generated *Hoxa4*-deficient mice and confirmed the downregulation of smooth muscle-specific contractile genes and the exacerbation of vascular remodeling after carotid artery ligation *in vivo*. Our results demonstrate HOXA4 is a novel repressor of VSMC phenotypic switching by inhibiting YAP/TEAD-mediated transcription.

Keywords: Hippo signaling/homeobox genes/phenotypic switching/vascular remodeling

1 **Introduction**

2 In spite of extensive effort for the improvement of therapeutic strategies, vascular
3 remodeling is still the major medical problem worldwide, which is closely related to the
4 pathogenesis of atherosclerosis, coronary heart diseases, peripheral artery diseases, and
5 pulmonary hypertension, indicating the need for more-detailed understanding of its etiology and
6 underlying molecular mechanisms.

7 Vascular remodeling is characterized by morphological and structural changes with
8 alterations in cell proliferation, cell migration, and cell death in the vasculature [1], which is
9 mainly composed of vascular smooth muscle cells (VSMCs), endothelial cells, and fibroblasts.

10 Among these cells, VSMCs are located in the midwall of the blood vessels and well-differentiated
11 in the quiescent state with high expression of contractile smooth muscle-specific genes such as
12 SM α -actin (α -SMA), smooth muscle 22 alpha (SM22 α), calponin, smoothelin, and SM myosin
13 heavy chain (Myh11) [2]. However, once exposed to inflammatory stimuli or vascular injury,
14 VSMCs transform from a contractile phenotype to a proliferative synthetic phenotype and
15 produce extracellular matrix and matrix metalloproteinases. This phenomenon is called
16 phenotypic switching, which contributes to the formation of vascular remodeling. The regulatory
17 gene programming during this process has been investigated intensively, and recent studies have
18 identified the Hippo signaling effector, Yes-associated protein (YAP) and the transcriptional
19 enhancer activator domain (TEAD) family, as a critical modulator of phenotypic switching [3-5].

20 The Hippo signaling pathway has been shown to play a central role in controlling organ
21 size by regulating cell proliferation, apoptosis, and stem cell programming [6]. This recently
22 discovered pathway was originally described in *Drosophila* [7] and later found to be conserved

1 in mammals [8]. The Hippo pathway consists of a two-step kinase cascade, in which mammalian
2 sterile 20-like 1/2 (MST1/2) and their scaffold protein salvador homolog 1 (SAV1) phosphorylate
3 large tumor suppressor 1/2 (LATS1/2), then LATS1/2 and their adaptor protein MOB kinase
4 activator 1A/B (MOB1A/B) phosphorylate YAP in response to various environmental cues such
5 as cell–cell contact [9], mechanotransduction [10], growth factors [11], and metabolic stress [12].
6 Phosphorylated YAP is inactivated with cytoplasmic retention by 14-3-3 proteins or proteasomal
7 degradation via ubiquitination [13]. On the other hand, unphosphorylated YAP translocates into
8 the nucleus, where it interacts with TEADs to promote downstream gene transcription required
9 for cell growth such as *CTGF* [14], *CYR61* [15] and ankyrin repeat domain 1 (*ANKRD1*) [16]. In
10 this transcriptional complex, YAP lacks a DNA-binding domain but works as a transcriptional
11 coactivator, whereas TEADs (TEAD1-4) are transcription factors that bind to the promoter
12 regions of their target genes via a GTIIC sequence (5'-GGAATG-3') [14,17].

13 Numerous studies have shown that inappropriate YAP/TEAD transactivation strongly
14 promotes cell proliferation and migration, followed by tissue overgrowth or tumorigenesis [6]. In
15 fact, YAP/TEAD transcriptional activation is a hallmark of many human cancers [18]. Recently,
16 the importance of YAP/TEAD-mediated gene regulation is recognized not only in the cancer field
17 but also in the cardiovascular field [19,20]. Although emerging evidence supports that the
18 YAP/TEAD plays an essential role in VSMC phenotypic switching and subsequent vascular
19 remodeling [3-5], downstream molecules that affect YAP/TEAD transcriptional activity remain
20 to be elucidated.

21 In the present study, we sought to discover a novel regulator of YAP/TEAD-dependent gene
22 expression. Using an unbiased shRNA screen, we detected Homeobox A4 (HOXA4) as a potent

1 repressor of YAP/TEAD-mediated transcription by competing with YAP for TEAD binding. We
2 also found that expression of HOXA4 is abundant in the vasculature, and HOXA4 maintains the
3 differentiation state of VSMCs via inhibition of YAP/TEAD-induced phenotypic switching,
4 which was confirmed by exacerbated vascular remodeling after carotid artery ligation in *Hoxa4*-
5 deficient mice. This study provides novel molecular insights into vascular remodeling.

Results

Pooled shRNA screening identified HOXA4 as a novel negative regulator of YAP/TEAD transcriptional activity

To investigate the transcriptional activity of YAP/TEAD, an 8×GTIIC-luciferase plasmid containing eight TEAD-DNA binding motifs is commonly used for cell-based reporter assays [10]. Based on this method, we first generated a fluorescent reporter cell line (8×GTIIC-EmGFP-293T; see Methods) to monitor YAP/TEAD-dependent transcriptional activity (Appendix Fig S1A). This cloned cell line mimicked the transcriptional activity of YAP/TEAD, because EmGFP intensity was faithfully dependent on cell culture density (Fig 1A) or highly responsive to disruption of LATS2, one of the main upstream Hippo pathway components, or forced expression of a constitutively active form of YAP (YAP-S127A) [9] (Fig 1B). We introduced a pooled lentiviral shRNA library consisting of 27,500 shRNAs targeting 5,043 genes (DECIPHER, Cellecta Inc.) into 8×GTIIC-EmGFP-293T cells at a MOI of 0.3. After cells were kept over-confluent for more than 2 days to maximally repress YAP/TEAD activity at baseline, cells expressing RFP (transduction marker) were sorted into two subpopulations by FACS, “GFP high” or “GFP low”, representing 5% or 50% of the cells with highest or lowest GFP signals, respectively. After genomic DNA extraction and PCR amplification, we sequenced shRNAs from the two cell populations using a next-generation sequencer (Fig 1C). Then we compared the abundance of each shRNA between these two subpopulations and focused on shRNAs targeting negative regulators of YAP/TEAD signaling that were enriched in the “GFP-high” sample. After quantile normalization (Appendix Fig S1B and C), the mean frequency of corresponding shRNAs towards each gene was assessed (Fig 1D). We selected 24 candidate genes whose knockdown

1 strongly changed EmGFP intensity as positive hits in the primary screen, and they were validated
2 using the 8×GT10C luciferase assay in HEK293T cells with a different sets of shRNAs (Appendix
3 Fig S1D). Because knockdown of HOXA4 upregulated luciferase intensity most significantly
4 (Appendix Fig S1D), we focused further efforts on HOXA4. HOXA4 knockdown in HEK 293T
5 cells not only upregulated luciferase activity but also resulted in the upregulation of *ANKRD1*
6 mRNA, one of the most well-known YAP/TEAD target genes [16] (Fig 1E and F). On the other
7 hand, forced expression of HOXA4 significantly suppressed *ANKRD1* expression (Fig 1G).
8 HOXA4 is a member of the homeobox gene family, which consists of 39 genes assigned to 13
9 paralog groups on four separate chromosomes. Homeobox genes have spatial and temporal
10 expression patterns during the embryonic period, in which they regulate the development of
11 various organs in corresponding somites as transcriptional factors [21]. Although the functions of
12 homeobox genes in adult have not been fully investigated, some homeobox genes were recently
13 reported to be expressed even in adult organisms and relate with various diseases [22,23]. To date,
14 the function of HOXA4 in YAP/TEAD-dependent gene regulation has not been described, but the
15 above data indicated that HOXA4 is a novel repressor of YAP/TEAD transcriptional activity.

16
17 *HOXA4 negatively regulates YAP/TEAD transcriptional activity independent of YAP*
18 *phosphorylation*

19 HOXA4 knockdown also resulted in the upregulation of other known YAP target genes,
20 such as *CTGF*, *CYR61*, and *BIRC5* (Fig 2A and Appendix Fig S2A) [24]. On the other hand,
21 forced expression of HOXA4 reduced the transcription of these genes except for *BIRC5*
22 (Appendix Fig S2B and C). We next determined whether HOXA4 acts through the canonical

Hippo kinases to regulate YAP. Because the expression of Hippo signaling kinases was unaffected by HOXA4 knockdown, these genes are not direct transcriptional targets of HOXA4 (Appendix Fig S3). HOXA4 reduced 8×GTIIC-luciferase reporter responses even in LATS1/2 knockdown cells; therefore, HOXA4 seemed to work in the downstream of the Hippo signaling cascade (Fig 2B). YAP with mutations in all five LATS kinase phosphorylation motifs (YAP-5SA) constitutively stayed in the nucleus and promoted the transcription of downstream genes [9]. Unexpectedly, forced HOXA4 expression significantly suppressed YAP-5SA-induced gene expression (Fig 2C). Moreover, knockdown of endogenous HOXA4 further promoted YAP-5SA-induced gene expression (Fig 2D). Therefore, the suppression of YAP/TEAD transcriptional activity by HOXA4 seemed to be entirely independent of YAP phosphorylation. We also confirmed that overexpression of HOXA4 significantly suppressed YAP target genes such as *CTGF* and *CYR61* (Fig EV1A) and that shHOXA4 significantly upregulated these genes (Fig EV1B). TAZ is a paralog of YAP [25]. TAZ target genes such as *CTGF* and *CYR61* were enhanced by shHOXA4 and suppressed by HOXA4 (Fig EV1C).

HOXA4 physically interacts with TEAD but not YAP

Several genes have been reported to inhibit YAP/TEAD activity via protein–protein interactions such as VGLL4-TEADs [26], HNF4α-TEAD4 [27], and RUNX3-TEAD4-YAP complexes [28]. Because HOXA4 persistently stays in the nucleus without affecting YAP subcellular localization (Fig EV1D and 1E), we speculated that HOXA4 might physically interact with TEADs or YAP. To examine this possibility, we performed several protein–protein interaction experiments. In HEK 293T cells co-transfected with tagged-HOXA4 and TEAD1 or

YAP, the co-immunoprecipitation (co-IP) results indicated that HOXA4 interacts with TEAD1 (Fig 3A, Fig EV2A), but not YAP (Fig 3B). HOXA4 also interacted with other three isoforms of TEAD (Fig EV2B and 2C). We also investigated the potential interaction between endogenous proteins in HEK 293T cells and endogenous immunoprecipitation of TEAD1 pulled-down HOXA4 and YAP (Fig 3C). To map the HOXA4-TEAD1 interaction domains, several truncated mutants of HOXA4 and TEAD1 were generated. Sequential co-IP assays revealed HOXA4 binds to TEAD1 via HOXA4 amino acids (a.a.) 216–274, which is homeodomain (Fig 3D), and the N terminal region of TEAD1 (Fig 3E). Finally, TEAD1 a.a. 30–135 was successfully co-IP by HOXA4 a.a. 216–274 (Fig 3F), whereas TEAD1 a.a. 30–102, only TEA domain, failed to do so (Fig 3G). Taken together, these results indicated that HOXA4 inhibits YAP/TEAD transcriptional activity via its interaction with TEADs (Fig 3H and I).

HOXA4 competes with YAP for binding to TEADs to suppress the expression of YAP/TEAD target genes

The interaction feature of HOXA4-TEADs implies two possible mechanisms for the inhibition of YAP/TEAD transcriptional activity. One is HOXA4 might compete with YAP for binding to TEADs, the other is HOXA4 might attenuate TEADs-DNA binding affinity. To verify these hypothesis, we performed several chromatin immunoprecipitation (ChIP) assays in HEK 293T cells and assessed whether HOXA4 affects the binding of YAP or TEAD4 to the target chromatin at the CTGF and CYR61 promoters [29]. Both TEAD4 and YAP occupation to these promoters were decreased by HOXA4, but disruption of YAP binding was predominant rather than TEAD4 (Fig 4A). Of note, this blocking effect on YAP was more remarkable with

1 high YAP activity (i.e. low cell density) than low YAP activity (i.e. high cell density). Moreover,
2 a competitive co-IP assay in HEK 293T cells showed HOXA4 and nuclear localized form of
3 YAP (YAP-S127A) competed with each other for binding to TEAD4 (Fig 4B). Together, these
4 results provide compelling evidence that HOXA4 competes with YAP for binding to TEADs to
5 inhibit the expression of YAP/TEAD target genes (Fig 4C).

6 7 *HOXA4 specifically represses YAP/TEAD transcriptional activity among HOX families*

8 From the result of the shRNA screen, activation of YAP/TEAD-dependent transcription
9 was highly specific for knockdown of HOXA4 compared with the knockdown of other HOX
10 genes included in the library (Fig EV3A). However, because HOX family members have strong
11 sequence conservation in the homeodomain, we wondered whether other HOX genes could
12 interact with TEADs. Although HOXA9 is linked to the susceptibility to atherosclerosis [22],
13 HOXA9 did not change YAP-5SA induced 8×GT1IC luciferase activity (Fig EV3B) or *CTGF*
14 expression (Fig EV3C). Furthermore, only a modest decline in *CTGF* and *CYR61* expression
15 induced by YAP-5SA was observed with the truncated form of HOXA4 including the
16 homeodomain (Fig EV3D). Together, these data indicate non-conserved regions other than the
17 homeodomain of HOXA4 is essential for the inhibition of YAP/TEAD dependent transcription.

18 19 *HOXA4 maintains the differentiation state in vascular smooth muscle cells by inhibiting* 20 *YAP/TEAD mediated phenotype switching*

21 To investigate HOXA4 function *in vivo*, we first examined the expression pattern of
22 *Hoxa4* in mice. *Hoxa4* was highly expressed in lung, white adipose tissue, brown adipose tissue,

1 carotid artery, and aorta (Fig 5A). There have been already some reports showing HOXA4 is
2 related to lung cancer development [30] or adipocyte differentiation [31], but the vascular
3 function of HOXA4 is largely unknown. FANTOM5 human gene expression datasets show high
4 expression levels of *HOXA4* in VSMCs as well as fetal organs (Fig EV4A) [32,33]. We also
5 confirmed the expression of HOXA4 in a human aortic tissue (Fig EV4B). VSMC phenotypic
6 switching from a differentiated contractile phenotype to a synthetic proliferative phenotype is a
7 hallmark of vascular diseases. Recently, it has been reported that the expression of YAP is
8 induced during VSMC phenotypic transformation, and YAP plays a critical role in both
9 promoting VSMC proliferation and inhibiting smooth muscle-specific gene expression [3,4].
10 Therefore, we hypothesized that HOXA4 might be involved in the maintenance of VSMC
11 differentiation via YAP/TEAD suppression.

12 In response to PDGF-BB, a growth factor known to induce VSMC transformation [34],
13 the expression of *HOXA4* was significantly downregulated in cultured aortic SMCs with a
14 reduction in differentiated VSMC marker gene expression both in human (Fig 5B) and in mouse
15 (Fig EV4C). This finding was consistent with previous findings of reduced *HOXA4* expression
16 in human VSMCs in response to other inflammation stimuli, interferon gamma and tumor
17 necrosis factor alpha [35]. Similar to other cell types, HOXA4 and TEAD were mainly localized
18 in the nucleus, whereas YAP and TAZ were located in both the nucleus and cytoplasm in human
19 VSMCs (Fig EV4D and 4E).

20 YAP in human primary aortic smooth muscle cells attenuated the expression of smooth
21 muscle contractile genes, including α -SMA, *SM22 α* , *Myh11*, and *calponin* in transcription levels
22 as described previously (Fig 5C). On the contrary, HOXA4 facilitated the expression of these

smooth muscle contractile genes (Fig 5C). On the other hand, mRNA expression of YAP/TEAD target genes, such as *CTGF*, *CYR61*, transforming growth factor beta 2 (*TGFB2*), [36] and Cyclin D1 (*CCND1*) [4], was attenuated by HOXA4 (Fig 5F). We conformed these changes of differentiated VSMC marker genes using the protein levels by shYAP/TAZ (Fig 5D) and shHOXA4 (Fig 5E). Furthermore, overexpression of HOXA4 significantly attenuated VSMC proliferation (Fig 5G and Fig EV4E), although silencing of endogenous HOXA4 resulted in enhanced rates of VSMC growth (Fig 5I and Fig EV4F). This negative effect of HOXA4 on the proliferative capacity of VSMCs was also confirmed by bromodeoxyuridine (BrdU) incorporation analysis (Fig 5H and 5J). These phenotypic changes induced by HOXA4 were completely the opposite to those by YAP.

Such opposing phenotypic effects in VSMCs induced by HOXA4 and YAP demonstrated that YAP/TEAD might be negatively regulated by HOXA4. To investigate whether YAP/TEAD is involved in HOXA4-mediated inhibition of VSMC phenotype switching, we performed knockdown experiments of TEAD or YAP combined with HOXA4. As well as previous investigation of rat VSMCs [5], TEAD1 is predominantly expressed among the four isoforms (Appendix Fig S4) in human VSMCs, then we utilized shRNA targeting common sequence in TEAD1, 3, and 4. Reduced smooth muscle-specific protein by knockdown of HOXA4 was rescued by concomitant TEAD knockdown (Fig 6A), and YAP knockdown also canceled the increased proliferation rate by HOXA4 abrogation (Fig 6B). Disruption of the TEAD-HOXA4 interaction in VSMCs by knockdown of HOXA4 significantly increased the amount of endogenous TEAD-YAP complexes (Fig 6C) and increased the occupation of HOXA4 on the TEAD-binding region in the promoters of *CTGF* and *CYR61* but not *α SMA* attenuated that of

phosphorylation-defective YAP (Fig 6D). Together, these data demonstrate that HOXA4 has a potent negative effect on VSMC phenotypic switching via YAP/TEAD suppression in the same manner identified in HEK 293T.

Loss of HOXA4 exacerbates vascular remodeling in mice

To clarify the pathophysiological function of HOXA4 in VSMCs, we created a *Hoxa4*-deficient mouse line using CRISPR-Cas9 gene editing (Appendix Fig S5A) [37]. The gene length of *Hoxa4* gene is 1.6 kilobase pairs and contains only two exons. In order to exclude the possibility that a functional truncated peptide was translated, we deleted almost all genomic regions of *Hoxa4* by inducing double-strand breaks at two sites around the start and stop codons and following homologous recombination repair (Appendix Fig S5B and 5C). YAP/TEAD is important for organ size control, but there was no significant difference in organ weight between *Hoxa4* KO- and WT-adult mice (Appendix Fig S6A). A staining for VSMCs and endothelial cells of *Hoxa4* KO embryos detected no morphological difference in the dorsal aorta compared with WT (Appendix Fig S6B). The contractibility of primary VSMCs harvested from *Hoxa4* KO and WT mice was also compared using a collagen gel assay and found to be almost similar (Appendix Fig S6B).

At baseline, there were very slight differences in smooth muscle marker gene expression in the carotid artery; however, YAP/TEAD transcriptional target genes related to cell growth are significantly upregulated in *Hoxa4* KO mice compared with WT mice (Fig 7A and 7D). The mouse carotid artery ligation model is commonly used for investigating vascular remodeling due to proliferation of phenotypically switched VSMCs [38]. Indeed, mice with genetic deletion

1 of YAP in VSMCs attenuate neointima formation in the proximal portion of the ligated carotid
2 artery [3], so we assessed vascular remodeling of *Hoxa4* KO mice with this experimental
3 model. Downregulations of differentiated smooth muscle genes are usually observed in 1 week
4 after ligation, much earlier than morphological remodeling occurs [5,38,39]. A more remarkable
5 reduction in smooth muscle marker gene expression was observed in *Hoxa4* KO mice compared
6 with WT mice (Fig 7B). Although we could not detect a specific signal of mouse *Hoxa4* protein
7 due to the absence of commercially available antibodies (See Methods), the transcription of
8 *Hoxa4* in the ligated carotid artery was significantly reduced at 1 week after operation (Fig 7C).
9 The predominant expression of YAP/TEAD target genes in the carotid arteries of *Hoxa4* KO
10 mice was also observed at 1 week after ligation (Fig 7E). These differences in differentiation- or
11 proliferation-associated genes in the injured artery between WT and *Hoxa4* KO mice were also
12 confirmed by immunoblotting (Fig 7F) or immunohistochemistry (Fig EV5A), leading to a two-
13 fold increase in neointima formation in *Hoxa4* KO mice at 250μm proximal to the ligation (Fig
14 7G to 7I) and at 500μm proximal to the ligation (Fig EV5B to 5D). Taken together, the above
15 data demonstrate that *Hoxa4* prevents VSMC phenotypic transformation and following vascular
16 remodeling after arterial ligation injury *in vivo*. Mechanistically, such phenotype is, at least in
17 part, due to YAP/TEAD suppression via TEAD-HOXA4 interaction as observed from *in vitro*
18 results.

Discussion

Here, by using a pooled shRNA screen, we demonstrated that HOXA4 is a novel negative modulator of YAP/TEAD transcriptional activity and also suppressed YAP/TEAD-induced phenotypic switching of VSMCs *in vitro* and *in vivo*. In spite of numerous discoveries of regulators of YAP/TEAD-mediated transcription that work upstream of the Hippo signaling cascade, few modulators have been detected to affect downstream molecular events. We first performed an unbiased screen using a pooled shRNA library and detected several candidate genes of putative YAP/TEAD repressors. Although the library does not include well-known negative regulators of YAP/TEAD such as cell surface proteins, shYAP transduced cells presented the lowest GFP intensity, and shRNAs targeting MAPK [40], ERBB4 [16], and Wnt [41] signaling pathway-related genes were accumulated in the “GFP low” population. These pathways are known to have a positive effect on YAP/TEAD transcriptional activity, which validated our initial screen. Meanwhile, in the “GFP high” population, shRNAs targeting ribosome biogenesis- or translation-related genes were enriched. These processes are fundamental biological functions of cells and affect many signals. Therefore, we excluded these genes as candidates.

HOXA4 is one of the HOX genes, which consist of 39 genes clustered into four chromosomes in human and mouse. Each HOX gene encodes a conserved DNA-binding motif, homeodomain of 61 amino acids, with spatial and temporal expression patterns during the embryonic period, where they work as a transcription factors and regulate the expression of downstream genes to build segments or structures in the body [21]. Therefore, mutations in HOX genes cause congenital disorders [42]. Recent studies have provided increasing evidence that HOX genes continue to be regionally expressed even in the adult organisms and related to disease

1 formation and development including cardiovascular diseases. For instance, HOXA9 has an
2 athero-protective effect via inhibiting NF- κ B-dependent gene expression in endothelial cells and
3 VSMCs [22]. HOXA5 is also an athero-protective gene that suppresses blood flow-dependent
4 endothelial inflammation [23].

5 To date, the molecular function of HOXA4 in adult organs is largely unknown. However,
6 HOXA4 has been reported as a tumor suppressor [43], and a recent study revealed its involvement
7 in lung cancer progression via inhibiting the Wnt- β catenin signaling by upregulation of GSK3 β
8 [30]. In fact, whole genome sequencing of young patients with non-small cell lung cancer detects
9 mutations of HOXA4 as well as those of MST1, the upstream kinase of the Hippo signaling
10 pathway, as cancer-associated gene mutations [44]. Although the molecular mechanisms of
11 HOXA4-mediated regulation of cell proliferation may be different among each cell type, Wnt-
12 signaling and Hippo signaling interact closely with each other [41,45], then further investigation
13 is needed to test the possible cross-talk between these two pathways in relation to HOXA4.

14 Mechanistically, HOXA4 inhibits YAP/TEAD transcriptional activity via HOXA4-TEADs
15 interaction. Because the HOXA4 interaction region in TEAD1 (residues 30–135) overlaps with
16 its DNA-recognition helix (residues 88–101) required for DNA binding of TEAD1 (Fig 3F) [46],
17 we first considered the possibility HOXA4-TEAD1 interaction might affect the DNA-binding
18 ability of TEAD1. However, HOXA4 had little effect on the DNA-TEAD affinity unexpectedly,
19 whereas a much more remarkable reduction in TEAD-mediated YAP-DNA binding was observed
20 (Fig 4A and 6D). Then, co-IP assays confirmed that HOXA4 competes with YAP for TEAD
21 binding. This finding is a reasonable explanation that the inhibition of YAP/TEAD transcriptional
22 activity was not seen in other HOX genes in spite of their binding abilities to TEAD via the

homeodomain. Together, these data indicated that non-conserved regions other than the homeodomain of HOXA4 mediate the inhibition of TEADs-YAP interactions. Recently, several molecules have been shown to suppress YAP/TEAD transcriptional activity via interaction with TEAD, such as VGLL-4 [26], RUNX3 [28], and HNF4 α [27]. However, the mechanisms of transcriptional suppression are completely different among these molecules including HOXA4. VGLL4 is a transcriptional cofactor without a DNA binding domain. The interaction region of TEADs with the TDU domain of VGLL4 overlaps the YAP-binding domain of TEADs, which inhibits the YAP-TEADs interaction [26]. On the other hand, RUNX3 and HNF4 α are transcription factors. RUNX3 binds to both TEAD4 and YAP, forming ternary complex of RUNX3-TEAD4-YAP. The interaction domain of TEAD4 with the Runt domain of RUNX3 overlaps with the DNA recognition helix (H3), and thus RUNX3 abrogates the ability of TEAD4 to bind DNA whereas RUNX3 does not affect the YAP-TEAD4 interaction [28]. HNF4 α associated with TEAD4 but not YAP. HNF4 α competes with YAP to interact with TEAD4, resulting in a reduction in YAP-DNA binding just like HOXA4. However, HNF4 α associates with both, but rather the N-terminal than the C-terminal regions of TEAD4 and does not change TEAD-DNA affinity [27], which are different from HOXA4.

In our study, *Hoxa4* KO mice show no apparent phenotype other than the previously reported mild skeletal alterations at baseline [47]. A possible explanation of this finding is that other Hox genes have functional redundancy in terms of development, which is supported by a previous report of embryonic lethality observed in *Hoxa4/Hoxb4* double mutant mice [48]. On the other hand, we found *Hoxa4* KO mice showed exacerbated neointima formation of the ligated carotid artery, in which YAP/TEAD transcriptional activity is highly upregulated (Fig 7).

1 This observation implies that HOXA4 has a specific molecular function and may be important
2 for the maintenance of homeostasis in YAP-activating conditions, such as cancer [18] or
3 vascular remodeling [3], because repression of the YAP-TEAD interaction by HOXA4 was only
4 observed with high YAP activity (i.e. low cell density) (Fig 4A).

5 YAP/TEAD play a critical role in the phenotypic switching of VSMCs and subsequent
6 vascular remodeling [3-5]. YAP/TEAD is also activated by well-known VSMC phenotypic
7 switch-inducing factors such as PDGF-BB [4], angiotensin II [49], and thromboxan-A2 [50].
8 Inhibition of YAP/TEAD activity alleviates VSMC phenotypic transformation *in vitro* and
9 vascular remodeling *in vivo*. Although upstream regulators of YAP/TEAD, such as Rho-actin
10 signaling followed by a change in YAP phosphorylation, is well investigated, [10,36,50] the
11 downstream molecules to affect YAP/TEAD activity in VSMCs remain to be elucidated. Previous
12 observations that downregulation of smooth muscle contractile genes is induced by YAP but not
13 YAP-S94A (TEAD-binding defective mutant) [5] and that upregulation of proliferative genes
14 such as *CTGF*, *CYR61*, *ANKRD1*, *Myc*, *TGFβ2*, and *CCND1* is inhibited by verteporfin (an
15 inhibitor of the binding of YAP and TEAD) [36,49] indicate phenotypic switching of VSMCs is
16 mediated by YAP/TEAD-dependent transcription. In this study, we detected HOXA4 as a novel
17 downstream repressor of YAP/TEAD transcriptional activity in VSMCs and maintenance of
18 HOXA4 expression could be a potential therapeutic strategy for vascular remodeling.

19 There were several limitations to this study. First, the function of HOXA4 as a
20 transcription factor is still unknown; therefore, the phenotype of human VSMCs or Hoxa4 KO
21 mice in our study might be partially affected by HOXA4 target genes. Second, it is uncertain
22 whether the phenotype of exacerbating neointima formation in *Hoxa4* KO mice is due to only

1 synthetic VSMCs or in conjunction with other cell types such as endothelial cells. These

2 questions are to be investigated further.

3 We conclude that HOXA4 maintains the differentiation of VSMCs by inhibiting
4 YAP/TEAD-induced VSMC phenotypic switching. These findings give us a better understanding
5 of the vascular pathophysiology and a novel therapeutic approach for vascular remodeling.

Materials and Methods

Plasmids

pENTR-3×FLAG-YAP-S127A (#42239) [51], pCMV-Flag-YAP-5SA/S94A (#33103) [14], 8×GTIIC-luciferase (#34615) [10], 3×Flag-pCMV5-TOPO TAZ-S89A (#24815) [52], Human HOXA9-MSCV-short (FLAG-tagged) (#20978) [53], Myc-TEAD4 (#24638) [54], and pCMX-Gal4-TEAD2 (#33107) [14] were obtained from Addgene. A wild-type (WT) Yes associated protein (YAP) construct was created from a YAP-S127A construct using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) in accordance with the manufacturer's instructions. A YAP-5SA construct was created from the YAP-5SA/S94A construct, and a WT transcriptional coactivator with PDZ-binding motif (TAZ) construct was created from the TAZ-S89A construct in the same way. Because large tumor suppressor kinase (LATS)-mediated phosphorylation of serine is critical for the inactivation of YAP in the cytoplasm, YAP^{serine 127→alanine} (S127A) or YAP^{serine 61, 109, 127, 164, 381→alanine} (5SA) mutants are constitutive active form of YAP, which remain in the nucleus [9].

Protein coding regions of human TEA domain transcription factor 1 (TEAD1), TEAD3, and Homeobox A4 (HOXA4) were subcloned from cDNA of HEK 293T cells. All constructs were correctly inserted into a lentiviral vector driven by a cytomegalovirus (CMV) promoter with 3×FLAG-tag or Myc-tag at the N-terminus.

Generation of 8×GTIIC-EmGFP-293T cell line

An 8×GTIIC-luciferase plasmid containing eight TEAD binding sites (CATTCCA) was obtained from Addgene (#34615) [10], and its luciferase sequence was changed to emerald

green fluorescent protein (EmGFP) subcloned from pcDNATM6.2-GW/EmGFP-miR vector (Invitrogen). Then 8×GTIIC-minimal promoter-EmGFP sequence was subcloned and introduced into a lentiviral vector without a promoter. After lentiviral transduction into HEK 293T cells, forty clones were obtained. Among them, a single clone that has the highest EmGFP reactivity to YAP-S127A, shLATS2, and cell density was isolated and named as 8×GTIIC-EmGFP 293T cell line.

ShRNA screening

Packaging for pooled lentiviral shRNA library (DECIPHERTM Human Module 1; Cellecta) was performed in accordance with the manufacturer's instructions and lentiviral particles were made. The library consists of 27,500 shRNAs targeting 5,043 genes with a puromycin-resistance marker and expression of tagged red fluorescent protein (RFP) fluorescent protein as transduction indicators. A total number of 8×10^6 8×GTIIC-EmGFP 293T cells were infected with the pooled lentiviral shRNA library at a multiplicity of infection (MOI) 0.3 to ensure that only one shRNA was introduced into each cell. The transduced cells were selected for 5 days with puromycin (1 µg/mL) with maintenance of a library coverage of more than 1,000 fold. After cells were maintained over-confluent for more than 48 hours to ensure that EmGFP intensity was maximumly suppressed, cells were sorted based on RFP using a fluorescence activated cell sorter (FACS) AriaTM III (BD Biosciences). Then, cells were sorted into two subpopulations, "GFP high" or "GFP low", representing 5% or 50% of the cells with highest or lowest GFP signals, respectively. Genomic DNA was extracted from a total of 5.5×10^5 cells in the "GFP high" population and 2.8×10^7 cells in the "GFP-low" population, and

shRNA-specific barcodes were PCR-amplified from genomic DNA using HT Sequencing Kits (Illumina) in accordance with the manufacturer's instructions. Both populations were then analyzed by Illumina sequencing, and the frequency of each shRNA was determined using Barcode Deconvolution software (Cellesta). After quantile normalization, the log₂ (lead abundance of top 5% GFP expressing population/that of bottom 50%) was detected.

Cell lines and transfection

HEK 293T cells were purchased from the American Type Cell Collection. Lipofection was used for DNA transduction to HEK 293T cells. Transfection of overexpression or shRNA-mediated knockdown vectors into HEK 293T cells was performed using polyethylenimine "MAX" (Polysciences), then cells were used for experiments 2 or 3 days later.

Dual luciferase assay

A total of 4×10^4 HEK 293T cells were seeded in 24-well plates and transfected with 0.4 µg of expression or knockdown vectors and 0.2 µg of 8×GT10C-luciferase with 0.005 µg of *Renilla* luciferase reporter by lipofection. Three days later, both luciferase activities were measured using a dual-luciferase reporter assay system (PicaGene dual kit; Toyo Ink).

Human and mouse primary vascular smooth muscle cells

Primary human aortic smooth muscle cells (Kurabo) were cultured in growth medium provided by the manufacturer and used for experiments at passage 5 (P5).

1 Mouse aortic smooth muscle cells were isolated from thoracic aortas of 8-week-old male
2 mice as described previously [55]. Briefly, the adventitia and endothelium were removed after
3 digestion with collagenase type II (175 units/mL; Worthington) for 20 minutes. Subsequently,
4 the media were digested with collagenase type II (175 units/mL) and elastase (0.5 mg/mL;
5 Sigma) for 45–60 minutes. Isolated cells were cultured on collagen type I-coated dishes
6 (IWAKI) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal
7 bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin at 37°C with 5%
8 CO₂, and then cells were used for experiments at passage 5 (P5).

9 For PDGF-BB stimulation, cells were seeded in 6-well plates at a concentration of
10 2.5×10⁵ cells per well, and serum starved with differentiation medium provided by the
11 manufacturer or DMEM containing 0.5% FBS for 24 hours, then subsequently stimulated with
12 10 ng/mL PDGF-BB (WAKO) for 24 hours.

14 *Lentivirus transfection*

15 Lentiviral transfection was used for DNA transduction into human VSMCs. Lentiviral
16 stocks were produced in HEK 293T cells as described previously [56-58]. In brief, virus-
17 containing medium was collected 48 hours post transfection and filtered through a 0.45-µm
18 filter. One round of lentiviral infection was performed by replacing the medium with virus-
19 containing medium (containing 8 µg/mL of polybrene), followed by centrifugation at 1,220 g
20 for 30 min at 32°C. Cells were used for analysis 3 days after DNA transduction. For knockdown
21 assays, lentiviral vectors coding shRNA designed by BLOCK-iT™ RNAi Designer (Invitrogen)
22 were used. The siRNA target sequences as follows [14]: siControl,

1 AAATGTACTGCGCGTGGAG; siHOXA4-1, CCAAGATGCGATCCTCCAA; siHOXA4-2,
2 GCAGAAGAAGACAGACCCT; siLATS1, GCAGCTGCCAGACCTATTA; siLATS2,
3 GGAAGATCCTCTACCAGAA; siSAV1, GGAAGATCCTCTACCAGAA; siYAP-1,
4 GACATCTTCTGGTCAGAGA; siYAP-2, CTGGTCAGAGATACTTCTT; siTAZ-1,
5 CCTCAATGGAGGGCCATAT; siTAZ-2, CCGTTTCCCTGATTTCCTT.

6 7 *Quantitative real-time PCR*

8 Total RNA was isolated using TriPure Isolation Reagent (Sigma-Aldrich) from cells or
9 tissues of mice using a homogenizer. Single-strand cDNA was synthesized from 100–1000 ng of
10 total RNA using a Verso cDNA synthesis kit (Thermo Fisher), and the products were analyzed
11 using a thermal cycler (StepOnePlus; Applied Biosystems) with THUNDERBIRD® SYBR
12 qPCR Mix (TOYOBO). Samples were normalized using β -actin mRNA expression in
13 experiments with HEK 293T cells or GAPDH mRNA expression in experiments with vascular
14 smooth muscle cells or mouse tissues.

15 16 *Protein extraction and western blotting*

17 Cultured cells or carotid arteries of mice were homogenized in lysis buffer consisting
18 of 100 mmol/L Tris-HCl, 75 mmol/L NaCl, and 1% Triton X-100 at pH 7.4 (Nacalai Tesque)
19 supplemented with Complete Mini protease inhibitor (Roche), 0.5 mmol/L NaF, and 10 μ mol/L
20 NaVO₄ just prior to use. Cytoplasmic and nuclear protein fractions were separated using NE-
21 PER™ (Thermo Fisher) in accordance with the manufacturer's instructions. A total of 20–30 μ g
22 (*in vitro*) or 5 μ g (*in vivo*) of protein was fractionated using NuPAGE 4–12% Bis- Tris gels

(Invitrogen) and transferred to a nitrocellulose membrane (Whatman). The membrane was blocked using phosphate-buffered saline (PBS) containing 5% nonfat milk for 30 minutes or Blocking One P (Nakarai Tesque) for 20 minutes and incubated with a primary antibody overnight at 4°C. After a washing step in PBS-0.05% Tween 20, the membrane was incubated with a secondary antibody for 1 hour at room temperature. The membrane was then washed and detected using PierceTM ECL or ECL Plus Western Blotting Substrate (GE Healthcare) with a LAS-4000 (Fuji Film). Quantification of western blots was performed using ImageJ64 software (NIH). Antibodies used are as follows: anti-TEADs (directed against the N-terminus of TEADs; 1:1000; CST #13295), anti-FLAG (1:1000; MBL PM020), anti-FLAG (1:1000; Sigma-Aldrich F1804), anti-Myc tag (1:1000; CST #2278), anti-Myc tag (1:5000; Sigma-Aldrich M4439), anti-HOXA4 (1:1000; Abcam ab131049), anti-YAP (1:1000; CST #14074), anti-TEAD1 (directed against the C-terminus of TEAD1; 1:5000; Abcam ab109080), anti- α SMA (1:1000; Sigma-Aldrich C6198), anti-SM22 α (1:400; Santa Cruz sc-53932), anti-TAZ (1:500; Sigma-Aldrich HPA007415), anti-YAP/TAZ (1:200; Santa Cruz sc-101199), anti-Calponin (1:200; Santa Cruz sc-58707), anti-PCNA (1:500; Santa Cruz sc-56), anti-Cyclin D1 (1:1000; Abcam ab92566), anti-CTGF (1:200; Santa Cruz sc-365970), anti-GAPDH (1:3000; CST #2118), anti-LAMIN A/C (1:400; Santa Cruz sc-6215), anti-Rabbit IgG, HRP-Linked (1:2000; GE NA934V), anti-Mouse IgG, HRP-Linked (1:2000, GE NA931V), anti-Goat IgG, HRP-Linked (1:2000, Abcam ab6885).

Co-immunoprecipitation assay

A total of $2.0\text{--}3.0 \times 10^6$ HEK 293T cells or human VSMCs were used for co-immunoprecipitation assays. The total cell lysate was incubated with the indicated antibodies and Dynabeads protein G (Thermo Fisher), or anti-FLAG antibody-conjugated magnetic beads (WAKO) in accordance with the manufacturer's instructions. Samples were extracted from magnetic beads using 2×LDS (two-fold dilution of NuPAGE™ 4× LDS Sample Buffer; Thermo Fisher) by boiling for 5 minutes at 95°C. The immune complexes were subjected to SDS-PAGE and analyzed by western blotting. Antibodies used are as follows: anti-TEAD1 (2μg; Santa Cruz sc-376113), normal mouse IgG1 (2μg, Santa Cruz sc-3877), anti-Myc tag (1:200, CST #2278), anti-FLAG (antibody-conjugated beads; WAKO 017-25151).

Chromatin immunoprecipitation

A total of 8.0×10^6 HEK 293T cells or human VSMCs were used for chromatin immunoprecipitation assays. For crosslinking, medium was replaced with 4.85 ml of DMEM supplemented with 140 μL of formaldehyde (FA) and incubated at room temperature for 10 minutes with rotation. After the medium was replaced with 5 mL of glycine solution (0.125 mol/L), cells were suspended with cold PBS and centrifuged. The supernatant was discarded and the crosslinked samples was homogenized in the hypotonic lysis buffer using a Dounce homogenizer supplemented with protease inhibitor cocktail, nuclease inhibitor, DTT, and NP40. The samples were then centrifuged at 10,500 g for 20 minutes at 4°C. The pellet was resuspended with immunoprecipitation buffer supplemented with protease inhibitor cocktail, nuclease inhibitor, dithiothreitol (DTT), and phenylmethylsulfonyl fluoride (PMSF), and chromatin was sheared by sonication (30 sec on and 30 sec off, 10 cycles) using a

BIORUPTOR® (Cosmo Bio). After the samples were centrifuged at 15,300 g for 10 minutes at 4°C, the supernatant (nuclear fraction) was divided and then antibodies for TEAD4 (1-2µg; Abcam ab58310), YAP (1-2µg; Abcam ab52771), TEAD1 (2µg; Santa Cruz sc-376113), normal mouse IgG1 (2µg, Santa Cruz sc-3877), FLAG-tag (2µg; Sigma-Aldrich F1804) or Myc-tag (2µg; Sigma-Aldrich M4439) were added. The mixtures of antibody and the nuclear extract were incubated overnight at 4°C with rotation, and then added to Dynabeads protein G and incubated for 2 hours at 4°C with rotation. One-tenth of the nuclear extract was retained as the input sample. After incubation, the supernatant was removed, and the beads were washed three times with immunoprecipitation buffer supplemented with the protease inhibitor cocktail, RNase inhibitor, DTT, and PMSF. The beads were then treated with DNA elution buffer supplemented with RNase H (New England BioLabs) and RNase A (Sigma-Aldrich) for 30 minutes at 37°C twice, followed by the treatment with Proteinase K (Sigma-Aldrich) for 1 hour at 65°C. After this, DNA was extracted and quantification of TEAD-binding sequences in *CTGF*, *CYR61* [29] or α -*SMA* [59] promoter was performed using quantitative real-time PCR. Primers used for ChIP assays are as follows [29]: human *CTGF* promoter forward, GCCAATGAGCTGAATGGAGT; reverse, CAATCCGGTGTGAGTTGATG; human *CYR61* promoter forward, CCCTTGCTGTTATGAGGAA; reverse, CCTTGCATTCCTTTGCATTT.; human α -*SMA* promoter forward, GGGACCTCAGCACAAAACCTC; reverse, GAAGGCTTGGCGTGTTTATC. A pair of primer for human *GAPDH* was used as a negative control [60]: forward, CCACATCGCTCAGACACCAT; reverse, CCCGCAAGGCTCGTAGAC.

Databases

Gene expression data in human various cell types or organs are obtained from RefEx (Reference Expression dataset: <http://refex.dbcls.jp/>) [32], an information browsing tool for Cap Analysis Gene Expression (CAGE) data from the RIKEN FANTOM 5 project (<http://fantom.gsc.riken.jp/5>) [33].

Immunocytochemistry

Human VSMCs or HEK 293T cells expressing HOXA4-GFP fusion protein were plated in a Nunc® Lab-Tek® II - CC2™ Chamber Slide™ (Sigma Aldrich) and then fixed in 4% paraformaldehyde (PFA) for 20 minutes at room temperature on the next day, followed by three washes in PBS. Then cells were permeabilized in 0.1% Triton for 10 minutes, followed by three washes in PBS. Subsequently, the cells were incubated in blocking buffer (5% donkey serum in PBS) for 15 minutes, and then incubated overnight at 4°C with primary antibodies in blocking buffer. After three washes in PBS, cells were incubated with secondary antibodies and DAPI (1 µg/mL) for 1 hour at room temperature. The slides were rinsed with PBS and mounted. Immunofluorescence was detected using an Axio Observer 7 (Zeiss). Primary antibodies used were as follows: anti-HOXA4 (1:100; Abcam ab131049), anti-GFP (1:50; Santa Cruz sc-9996), anti-YAP (1:100; CST #14074).

Cell viability assay

For proliferation assays, cells were trypsinized at 2 days after lentiviral infection and plated on 60-mm dish (5 mL) or in a 96-well plate (100 µL) at a concentration of 2×10^4

cells/mL. Cell numbers were counted using Countess II FL (Thermo Fisher) and cell viability was measured using Cell Counting Kit-8 (Dojindo) at the indicated time points.

BrdU incorporation assay

In vitro bromodeoxyuridine (BrdU) incorporation was performed to analyze cell proliferation. Human VSMCs were plated in a Nunc® Lab-Tek® II - CC2™ Chamber Slide™ (Sigma Aldrich) at a density of 1.0×10^4 cells/cm² at 2 days after transduction of indicated lentiviral vectors. The lentivirus containing medium was diluted twice for overexpression experiments due to high cell toxicity compared with the knockdown experiments. Another 2 days later, cells were stimulated with 10μM BrdU (Sigma, B9285) for 4 hours and fixed with 4% paraformaldehyde (PFA) for 30 minutes at 4°C. The slides were autoclaved (90°C, 20 min) in HistoVT One (Nakarai Tesque) for heat-mediated antigen retrieval. Then cells were permeabilized in 1.0% Triton for 15 minutes and blocked in 5% donkey serum/PBS for 15 minutes, followed by incubation with an anti-BrdU antibody (Abcam, ab6326, 1:250 dilution) at 4°C overnight. The slides were rinsed three times and then incubated with donkey anti-rat IgG secondary antibody Alexa Fluor 594 (1:200; Thermo Fisher A11007) with DAPI (0.5μg/mL) at room temperature for 1 h. The slides were washed and mounted in VECTASHIELD® Mounting Medium (Vector Laboratories, H-1000), and then observed using an Axio Observer 7 (Zeiss) with a 10× objective. Images were randomly acquired and the percentage of BrdU positive cells in each image was counted using ImageJ64 for analysis.

Collagen gel contraction assay

Collagen gel contraction assays were performed using a Collagen-based Cell Contraction Assay kit (Funakoshi Co., Ltd.) in accordance with the manufacturer's instructions. Briefly, primary aortic smooth muscle cells were harvested from 8-weeks old male mice. A concentration of 4×10^5 cells/mL cell-collagen mixture was placed in a 24-well plate in a volume of 500 μ L. After gelation for 1 hour at 37°C, 1.0 mL of culture medium was added and the plate incubated for 48 hours. After the gels were gently released from each well, the size of the gel was sequentially photographed at the indicated time points and analyzed using ImageJ64 software.

Human samples of aorta

Human aortic samples were obtained from patients with abdominal aortic aneurysm (AAA) who underwent the artificial blood vessel replacement surgery with prior informed consent and written permission. Tissues were immediately fixed with 4% PFA for 48 hours and embedded in paraffin.

Mice

Cas9 mRNA and single guide RNAs (sgRNAs) were synthesized *in vitro* using a Transfection-ready Cas9 Smart Nuclease mRNA Kit (System Biosciences) and a Linearized T7 gRNA SmartNuclease vector Kit (System Biosciences) in accordance with the manufacturer's instructions. The following sequences were used for sgRNA synthesis: left sgRNA, GAACTTGGGCTCGATGTAGT; right sgRNA, CTCCATATAATCTAGAGACC.

Hoxa4 knockout (KO) mice were generated using the CRISPR/Cas9 system, as described previously [37]. In brief, Cas9 mRNA and sgRNAs were microinjected into fertilized embryos of C57BL/6J mice to induce subsequent non-homologous end joining. Mutations in the *Hoxa4* allele were confirmed by Sanger sequencing analyses (ABI 3100 genetic analyzer). All mice were genotyped within 4 weeks after birth, using PCR with specific primers as follows: mouse *Hoxa4* forward, ACGACACCGCGAGAAAAATTAG; mouse *Hoxa4* reverse, CTGTGCCCCAGTATAAGACAAC. Homozygous KO mice were generated from a heterozygous intercross and WT littermates were used as a control. All *in vivo* experiments were conducted using 8-week-old male mice with a C57BL/J background, except for measurement of body/organ weight (9-week-old male mice) and immunohistochemistry of embryos (embryonic day [E] 16.5). E0.5 was defined the day that vaginal plug was detected.

Mice were maintained in specific pathogen-free conditions at the Institute of Laboratory Animals of Kyoto University Graduate School of Medicine.

Carotid artery ligation model

Carotid artery ligation was performed as described previously [3,38]. In brief, mice were anesthetized with a mixture of medetomidine, midazolam, and butorphanol, then the left common carotid artery was exposed and completely ligated just proximal to the carotid bifurcation with 7-0 silk suture. Then, carotid arteries were harvested at 7 days after surgery for the extraction of RNA and protein or 28 days after surgery for histological analysis.

Histological analysis and immunohistochemistry

For the analysis of mouse carotid arteries, mice were perfused from the left ventricle with PBS followed by 4% PFA. The injured (left) and contralateral uninjured (right) arteries were dissected, fixed with 4% PFA and embedded in paraffin. Cross sections of the bilateral carotid arteries were stained with hematoxylin and eosin (HE) and Elastica Van Gieson (EVG). Neointima formation as well as vessel and media area were assessed at two different portions, 250 μ m and 500 μ m proximal to the ligature, using ImageJ64 software (NIH). Sections were also obtained from injured carotid arteries at 1,000 μ m for immunohistochemistry, then the samples were immunolabeled with an Alexa Fluor 488-conjugated anti-PCNA antibody (Abcam, ab201672, 1:50 dilution) and an anti-CTGF antibody (Abcam, ab6992, 1:50 dilution) followed by anti-rabbit IgG secondary antibody Alexa Fluor 594 and DAPI.

For the analysis of mouse embryos, embryos were euthanized by decapitation, fixed with 4% PFA for 24 hours and an additional 48 hours after the trunk was cut at the level between neck and the root of forearms, then embedded in paraffin. Cross sections of dorsal aorta were immunolabeled with a Cy3-conjugated anti- α SMA antibody (1:200; Sigma-Aldrich C6198) and an anti-CD31 antibody (1:50; Abcam ab28364) followed by anti-rabbit IgG secondary antibody Alexa Fluor 488 and DAPI.

For the analysis of human tissue, AAA samples were immunolabeled with a Cy3-conjugated anti- α SMA antibody (1:200; Sigma-Aldrich C6198) and an anti-HOXA4 antibody (1:100; Abcam ab28364) followed by anti-rabbit IgG secondary antibody Alexa Fluor 488 and DAPI. Images were acquired from relatively the healthy, marginal zone of AAA. Antigen retrieval using HistoVT One and blocking using Blocking One Histo (Nakarai Tesque) were

performed in accordance with the manufacturer's instructions after deparaffinization in all studies described above.

Antibodies

Although all commercially-available antibodies (Abcam ab131049, Abcam ab 26097, Santa Cruz sc-398426, Santa Cruz sc-515418, MyBiosource MBS9206995, MyBiosource MBS2525887, Biorbyt orb157564) that are described as being applicable for immunoblotting and/or immunohistochemistry of mouse origin in their datasheets were tried to detect mouse *Hoxa4*, no specific signal was observed in comparisons between WT- and *Hoxa4* deficient mice-derived samples. For the detection of human HOXA4, Abcam ab131049 was validated for immunoblotting or immunohistochemistry by knockdown and overexpression of HOXA4 in HEK 293T cells and human VSMCs.

Statistical analysis

Data are presented as means $\pm$ standard error of the mean (SEM). For statistical comparisons, unpaired Student's t-test (two groups, parametric), Mann-Whitney test (two groups, non-parametric), one-way analysis of variance (ANOVA) with Sidak's post-hoc test (three or more groups, parametric), or two-way ANOVA with Sidak's post-hoc test (two different categorical variables, parametric) were used as indicated in the Figure legends. A P-value <0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc.).

Study approval

All animal experiments were approved by Kyoto University Ethics Review Board and Animal Research Committee of Kyoto University. The study using human samples was approved by Institutional Review Board/Ethics Committee of the Kyoto University Graduate School of Medicine (No. G473) and conducted under the tenets of the Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

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Author contributions

KO and MK designed experiments. MK performed animal experiments with the help of TH, YI, ST, and YM. MK and OB performed FACS studies. MK performed other *in vitro* experiments with the help of RRR, TW, TY, CO and SX. MK, YN, TK and KO analyzed the data and prepared the manuscript. All authors discussed and commented on the manuscript.

Conflict of interest

None.

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Figure Legends

Figure 1 - Identification of HOXA4 as a negative regulator of YAP/TEAD transcriptional activity through a pooled shRNA screen.

A, Dynamic fluorescence patterns according to cell density in 8×GTIIC-EmGFP 293T cells.

B, EmGFP intensity in LATS2 knockdown, or forced expression of a constitutively active form of YAP (YAP-S127A). Scale bars indicate 100 μ m.

C, Schematic drawing of FACS-based pooled shRNA screen of molecules that affect the YAP/TEAD signaling pathway in 8×GTIIC-EmGFP 293T cells.

D, Scatter plots of mean frequency of each shRNA per gene. Average of v HOXA4 is indicated as a red dot.

E, Augmented 8×GTIIC-luciferase reporter activity by loss of HOXA4. ShSAV1 was used as an experimental control. **P<0.01 vs shCtrl, by ANOVA with Sidak's correction.

F and G, Quantitative real-time PCR analysis of *ANKRD1* expression in HEK 293T cells transfected with shHOXA4 (F) or HOXA4 expression vector (G). **P<0.01, by unpaired two-tailed Student's t-test.

Data information: Graphs of (E-F) show mean $\pm$ SEM; three biological repeats.

Figure 2 - HOXA4 negatively regulates YAP/TEAD transcriptional activity independently of YAP phosphorylation.

A, Quantitative real-time PCR analysis of *CTGF*, *CYR61*, and *BIRC5* in HEK 293T cells with knockdown of endogenous HOXA4. ShLATS1/2 and shYAP were used as an experimental control. *P<0.05, **P<0.01 vs shCtrl, by ANOVA with Sidak's correction.

B, 8×GT10C-luciferase reporter assays in HEK 293T cells transfected HOXA4 and/or shLATS1/2.

****P**<0.01 vs shCtrl, by two-way ANOVA with Sidak's correction.

C, Quantitative real-time PCR analysis of *HOXA4*, *YAP*, *CTGF*, and *CYR61* in HEK 293T cells

with overexpression of HOXA4 and/or constitutive active form of YAP (YAP -5SA). *P<0.05,

****P**<0.01 vs YAP-5SA with LacZ, by ANOVA with Sidak's correction.

D, Quantitative real-time PCR analysis of *HOXA4*, *YAP*, *CTGF*, and *CYR61* in HEK 293T cells

with knockdown of endogenous HOXA4 and/or overexpression of YAP-5SA. *P<0.05, ****P**<0.01

vs YAP-5SA with shCtrl, by ANOVA with Sidak's correction.

Data information: All data are presented as mean ± SEM; three biological repeats.

Figure 3 - Protein-protein interactions between HoxA4 and TEADs.

A and **B**, Association of HOXA4 with TEAD1 (A) but not YAP (B) indicated by co-IP experiments.

HEK 293T cells were co-transfected with FLAG-HOXA4 and Myc-TEAD1 or Myc-YAP.

C, Interaction between endogenous TEAD1 and HOXA4 by co-IP experiments in HEK 293T

cells. The interaction between endogenous TEAD1 and YAP was used as a positive control.

D, Immunoprecipitation using FLAG antibody in lysates from HEK 293T cells transfected with

plasmids expressing HOXA4 truncation mutants and TEAD1.

E, Immunoprecipitation using FLAG antibody in lysates from HEK 293T cells transfected with

plasmids expressing TEAD1 truncation mutants and HOXA4. Arrowheads indicate IgG heavy

chain. Arrows indicate TEAD1 (a.a. 1–426).

F, Requirement of TEAD1 (a.a. 30–135) for its interaction with HOXA4 via its homeodomain

(a.a. 216–274).

1 **G**, Failure of TEAD1 (a.a. 30–102), TEA domain alone, to interact with HOXA4.

2 **H** and **I**, Diagram showing the structure of truncated HOXA4 (**H**) and TEAD1 (**I**) constructs and
3 possible protein–protein interactions between them. Conservation between human TEAD1, 2, 3,
4 and 4 is shown (**I**).

5 Data information: All experiments above were repeated at least twice.

6
7 **Figure 4 - HOXA4 negatively regulates TEAD-YAP interaction.**

8 **A**, ChIP assays of CTGF and CYR61 promoters using TEAD4 or YAP antibodies in HEK 293T
9 cells transfected with plasmids expressing HOXA4. * $P < 0.05$, ** $P < 0.01$, by unpaired two-tailed
10 Student's t-test. Data are expressed as the means $\pm$ SEM of three independent experiments.

11 **B**, Competitive co-IP assay and densitometry showing significantly reduced TEAD-YAP
12 interaction by overexpression of HOXA4 in a dose-dependent manner in HEK 293T cells.
13 * $P < 0.05$, ** $P < 0.01$, by ANOVA with Sidak's correction. Data are presented as mean $\pm$ SEM of
14 three independent experiments.

15 **C**, Working hypothesis whereby HOXA4 inhibits YAP/TEAD-mediated transcription activity.

16
17 **Figure 5 - HOXA4 regulates the phenotypic switching of vascular smooth muscle cells.**

18 **A**, Expression of *Hoxa4* in various mouse tissues using qRT-PCR. Expression of glyceraldehyde-
19 3-phosphate dehydrogenase (*Gapdh*) mRNA was used as an internal control. WAT; white adipose
20 tissue, BAT; brown adipose tissue, Skm; skeletal muscle.

21 **B**. Quantitative real-time PCR analysis of differentiated VSMC marker genes in primary human
22 VSMCs with PDGF-BB stimulation for 24 hours. * $P < 0.05$, ** $P < 0.01$, by unpaired two-tailed

Student's t-test.

C, Quantitative real-time PCR analysis of smooth muscle specific genes in primary human VSMCs transduced with lentiviral vectors encoding overexpression or knockdown of HOXA4 and YAP. Cells infected with lentiviral vectors encoding LacZ or shCtrl were used as controls. *P<0.05, **P<0.01, by unpaired two-tailed Student's t-test.

D and **E**, Representative western blotting analysis of total protein lysates of primary human VSMCs transduced with lentiviral vectors encoding knockdown of YAP/TAZ (**D**) or HOXA4 (**E**). Representative western blotting analysis of total protein lysates of primary human VSMCs transduced with lentiviral vectors encoding knockdown of HOXA4.

F, Quantitative real-time PCR analysis of YAP/TEAD target genes in primary human VSMCs transduced with lentiviral vectors encoding overexpression or knockdown of HOXA4 and YAP. The cells infected with lentiviral vectors encoding LacZ or shCtrl were used as controls. *P<0.05, **P<0.01, by unpaired two-tailed Student's t-test.

G, Proliferation assay in VSMCs transduced with the indicated genes. Virus-transfected human VSMCs were plated at equal density in growth medium, and then cells were collected and counted at each time point as indicated. **P<0.01 vs LacZ, by ANOVA with Sidak's correction.

H, BrdU incorporation assays in VSMCs transduced with the indicated genes. The percentage of BrdU positive cells in each group is shown. Nine to twelve images were randomly acquired and analyzed. **P<0.01 vs LacZ, by ANOVA with Sidak's correction.

I, Proliferation assay in VSMCs transduced with the indicated genes. Virus-transfected human VSMCs were plated at equal density in growth medium, and then cells were collected and counted at each time point as indicated. *P<0.05 vs shCtrl, by ANOVA with Sidak's correction.

J, BrdU incorporation assays in VSMCs transduced with the indicated genes. The percentage of BrdU positive cells in each group is shown. Nine to twelve images were randomly acquired and analyzed. **P<0.01 vs shCtrl, by ANOVA with Sidak's correction.

Data information: Graphs are presented as means $\pm$ SEM; three biological repeats, except for (H) and (J).

Figure 6 - A negative effect of HOXA4 on VSMC phenotypic switching is mediated by YAP/TEAD.

A, Representative western blotting analysis of total protein lysates of primary human VSMCs transduced with lentiviral vectors encoding shRNAs of HOXA4 and TEAD1/3/4. Quantification of alpha smooth muscle actin and calponin protein levels is expressed as the means $\pm$ SEM of three independent experiments. *P<0.05, **P<0.01, by ANOVA with Sidak's correction.

B, Proliferation assay in VSMCs transduced with indicated genes. Virus-transfected human VSMCs were plated at equal density in growth medium, and then cell viability was analyzed by Cell Counting Kit-8 at indicated days at each time point as indicated. *P<0.05 vs shCtrl, by ANOVA with Sidak's correction. Data are presented as mean $\pm$ SEM of three independent experiments.

C, Representative immunoblotting images and quantification of the interaction between endogenous TEAD1 and HOXA4 or YAP using co-IP experiments in human VSMCs with knockdown of HOXA4. *P<0.05 vs shCtrl, by ANOVA with Sidak's correction. Data are presented as mean $\pm$ SEM of three independent experiments.

D, ChIP assays of CTGF, CYR61 and α -SMA promoters using anti-FLAG-tag, anti-Myc-tag, anti-TEAD1 antibodies in human VSMCs transfected with plasmids expressing Myc-tagged YAP-5SA and/or FLAG-tagged HOXA4. *P<0.05, **P<0.01 vs FLAG-GFP, by unpaired two-tailed Student's t-test. Data are expressed as the means $\pm$ SEM of three independent experiments.

Figure 7 - Hoxa4 KO mice alleviate smooth muscle phenotypic modulation and abrogate neointima formation after carotid artery ligation injury.

A, Quantitative real-time PCR analysis of differentiated smooth muscle marker genes in the left carotid artery of 8-week-old WT and *Hoxa4* KO mice (WT: n=7, *Hoxa4* KO: n=5).

B, Quantitative real-time PCR analysis of differentiated smooth muscle marker genes in the ligated left carotid artery of WT and *Hoxa4* KO mice at 1 week after ligation (n=6). Mean expression level of six WT mice at the baseline was defined as 1.0.

C, *Hoxa4* expression levels in the ligated left carotid artery of WT mice at 1 week after ligation (n=6).

D, Quantitative real-time PCR analysis of Yap/Tead target genes in the left carotid artery of 8-week-old WT and *Hoxa4* KO mice (WT: n=7, *Hoxa4* KO: n=5).

E, Quantitative real-time PCR analysis of Yap/Tead target genes in the ligated left carotid artery of WT and *Hoxa4* KO mice at 1 week after ligation (n=6). Mean expression level of six WT mice at the baseline was defined as 1.0.

F, Representative western blotting analysis and densitometry of non-ligated (right; R) and ligated (left; L) carotid arteries from WT and *Hoxa4* KO mice at 1 week after ligation (WT: n=6, *Hoxa4* KO: n=7). *P<0.05, **P<0.01, by two-way ANOVA with Sidak's correction.

G to I, Representative images showing Elastica van Gieson staining (G), and vessel, neointima and media area (H), and neointima to media layer ratio (I) of carotid arteries of WT and *Hoxa4* KO mice at 4 weeks after ligation (WT: n=14, *Hoxa4* KO: n=11).

Data information: Images and data were obtained at 250µm proximal to the ligation. Scale bars indicate 100 µm. *P<0.05, **P<0.01 vs WT, except in (C) vs baseline, by unpaired two-tailed Mann–Whitney test. Data are presented as mean ± SEM. N.D.; not detected.

Expanded View Figure Legends

Expanded View Figure 1 - HOXA4 negatively regulates YAP and TAZ-mediated TEAD transcriptional activity.

A, Quantitative real-time PCR analysis of *HOXA4*, *YAP*, *CTGF*, and *CYR61* in HEK 293T cells with overexpression of HOXA4 and WT YAP. **P<0.01 vs YAP overexpression with LacZ, by ANOVA with Sidak's correction. Data are presented as mean $\pm$ SEM of three independent experiments.

B, Quantitative real-time PCR analysis of *HOXA4*, *YAP*, *CTGF*, and *CYR61* in HEK 293T cells with knockdown of endogenous HOXA4 and overexpression of WT YAP. *P<0.05, **P<0.01 vs YAP overexpression with shCtrl, by ANOVA with Sidak's correction. Data are presented as mean $\pm$ SEM of three independent experiments.

C, Quantitative real-time PCR analysis of *CTGF* and *CYR61* in HEK 293T cells with knockdown or overexpression of HOXA4 and overexpression of TAZ. **P<0.01 vs TAZ overexpression, by ANOVA with Sidak's correction. Data are presented as mean $\pm$ SEM of three independent experiments.

D, Fluorescent images of HEK 293T cells transfected with GFP-HOXA4 fusion protein under different cell density. Scale bars indicate 50 μ m.

E, Representative immunofluorescent staining images for YAP, GFP, DAPI on HEK 293T cells transfected with GFP-HOXA4 fusion protein under different cell density. Scale bars indicate 50 μ m.

Expanded View Figure 2 - HOXA4 interacts with TEADs in HEK 293T cells.

A, Specific binding between TEAD1 and HOXA4. HEK 293 cells were transfected with Myc-TEAD1 and FLAG-HOXA4. The specificity of binding was confirmed by precipitation with anti-Myc antibody and further immunoblotting with anti-FLAG antibody.

B and C, Co-IP experiments detecting the interaction between HOXA4 and all isoforms of TEADs.

Data information: All experiments above were repeated at least twice.

Expanded View Figure 3 - Suppression of YAP/TEAD transcriptional activity is specific for HOXA4.

A, Distribution of the frequency of each shRNA targeting HOX family genes in shRNA screen. Log₂ (fold change) of each shRNA abundance in the “GFP-high” pool compared with the “GFP-low” pool is shown. Each dot represents different shRNA and vertical solid line represents the mean value. Three to five shRNAs against each HOX gene are contained in the library.

B and C, Luciferase reporter assay using 8xGTIIC-luciferase (B) and quantitative real-time PCR of *CTGF* (C) in HEK 293T cells with overexpression of YAP-5SA and HOXA4 or HOXA9.

D, Quantitative real-time PCR analysis of *CTGF* and *CYR61* in HEK 293T cells with overexpression of YAP-5SA and truncated mutants of HOXA4.

Data information: All data except for (A) are presented as mean ± SEM; three biological repeats.

****P<0.01** vs LacZ with YAP-5SA, by ANOVA with Sidak’s correction.

Expanded View Figure 4 - Cellular function of HOXA4 in vascular smooth muscle cells.

A, FANTOM5 CAGE data showing higher expression levels of HOXA4 in human VSMCs.

B, Representative immunofluorescent staining images for α -SMA and HOXA4 on human aortic samples. Scale bars indicate 20 μ m.

C, Expression of *Hoxa4* and markers of VSMC proliferation and contractile phenotype in mouse primary aortic smooth muscle cells stimulated with PDGF-BB. All data are presented as mean $\pm$ SEM of three independent experiments. *P<0.05, **P<0.01, by unpaired two-tailed Student's t-test.

D, Western blotting analysis of cytosolic and nuclear protein levels of HOXA4, YAP, TAZ, and TEAD1 in human VSMCs.

E, Immunocytochemistry of human VSMCs with anti-HOXA4 antibody. Scale bars indicate 50 μ m.

F and G, Representative images of BrdU incorporation assay in human VSMCs transduced with the indicated genes (related to Figure 6H and 6J). Scale bars indicate 200 μ m.

Expanded View Figure 5 - Neointima formation in *Hoxa4* deficient mice.

A, Representative immunofluorescent staining images for CTGF and PCNA on the neointima in left carotid arteries at 4 weeks after ligation. Sections are obtained at 1,000 μ m proximal to the ligation. Scale bars indicate 20 μ m.

B, Representative images showing EVG staining of left carotid arteries of WT and *Hoxa4* KO mice at 4 weeks after ligation. Sections are obtained at 500 μ m proximal to the ligation. Scale bars indicate 100 μ m.

C, Vessel, neointima and media area measured at 500 μ m proximal to the ligation. Age- and sex-matched WT (n=15) or *Hoxa4* KO (n=12) mice were subjected to carotid artery ligation. *P<0.05,

1 **P<0.01 vs WT, by unpaired two-tailed Mann–Whitney test. Data are presented as mean ± SEM.
2 **D**, Neointima to media layer ratio measured at 500 μm proximal to the ligation. Age- and sex-
3 matched WT (n=15) or *Hoxa4* KO (n=12) mice were subjected to carotid artery ligation. *P<0.05,
4 by unpaired two-tailed Mann–Whitney test. Data are presented as mean ± SEM.
5
6

Figure 1

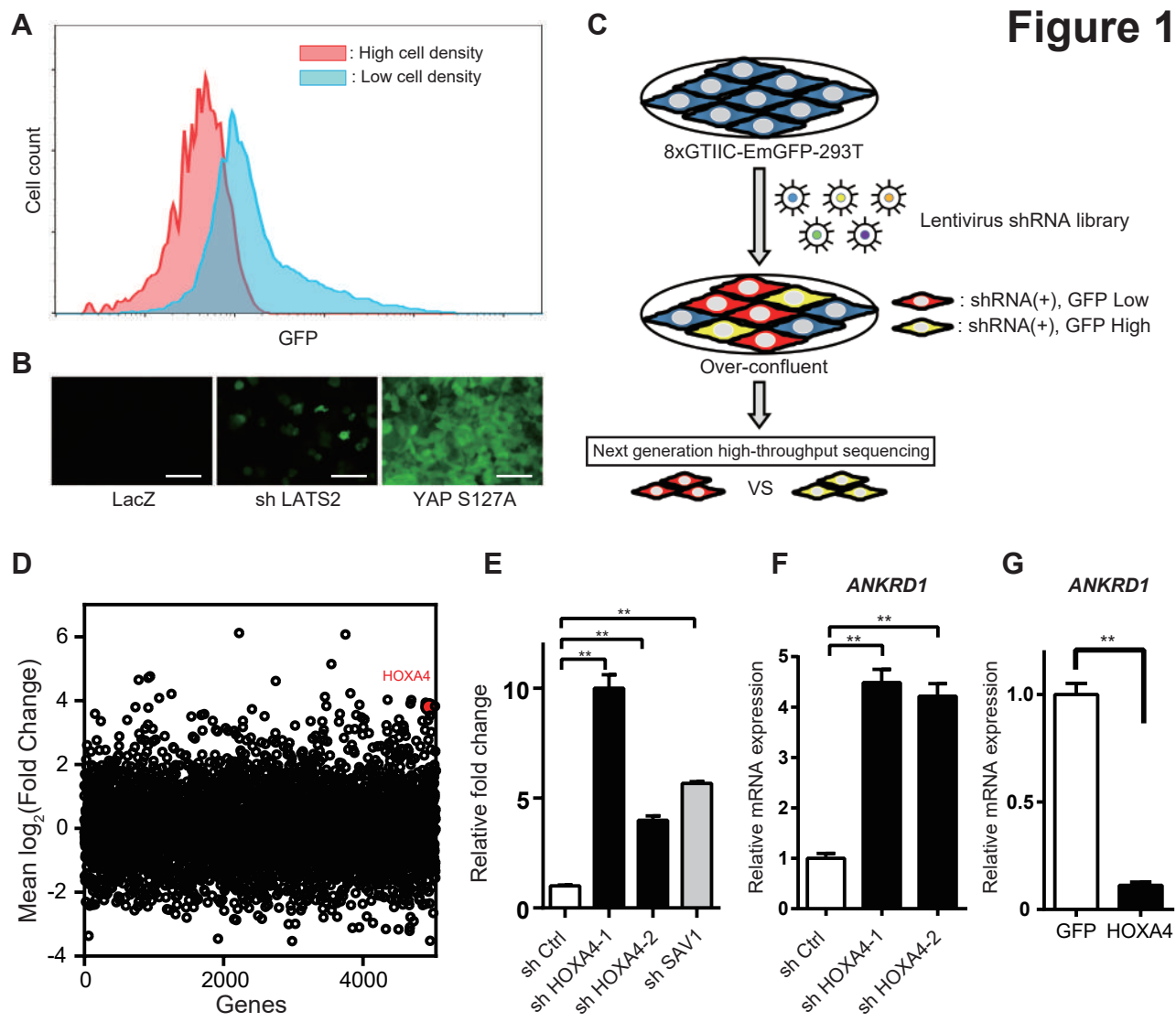


Figure 2

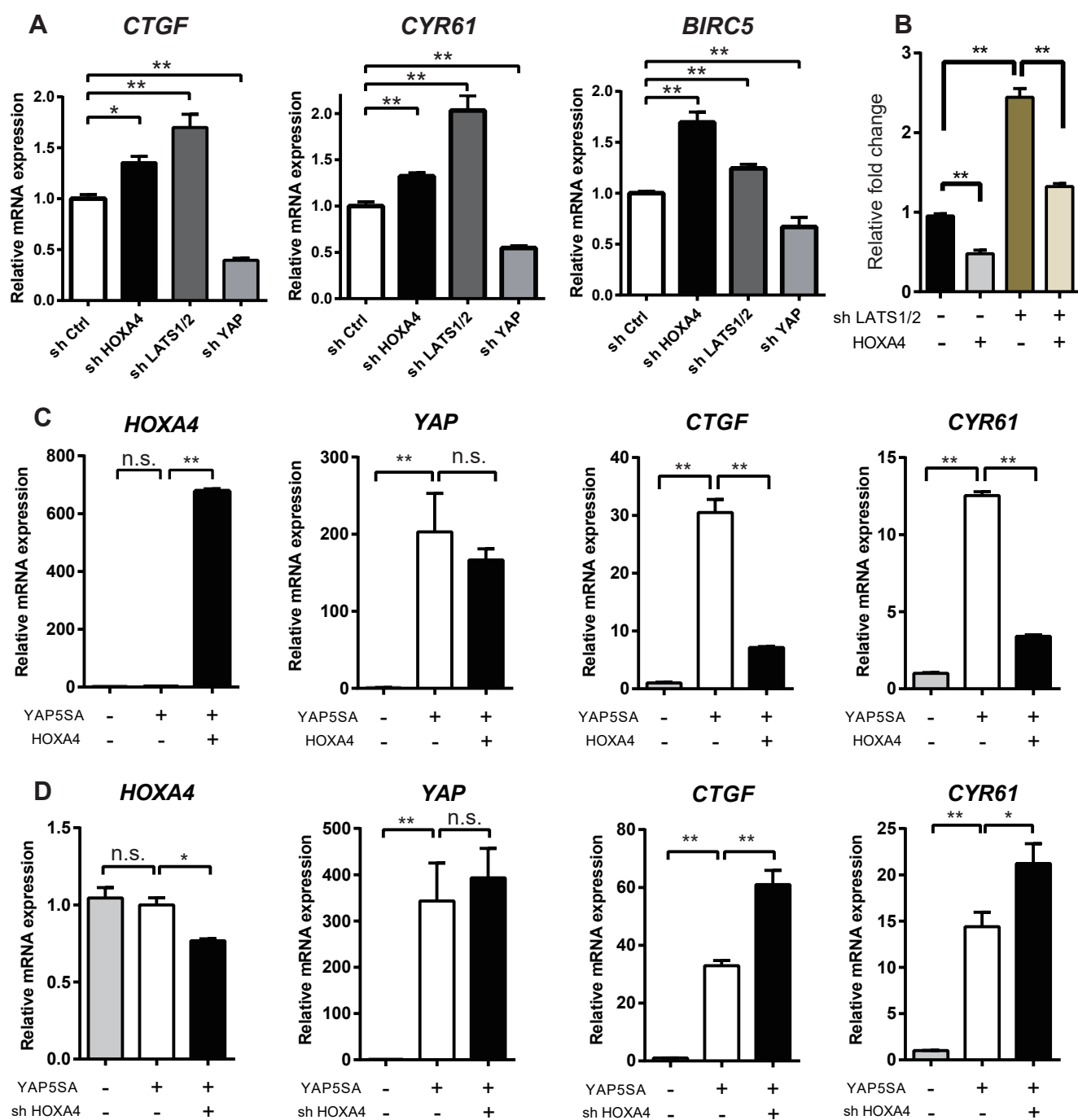


Figure 3

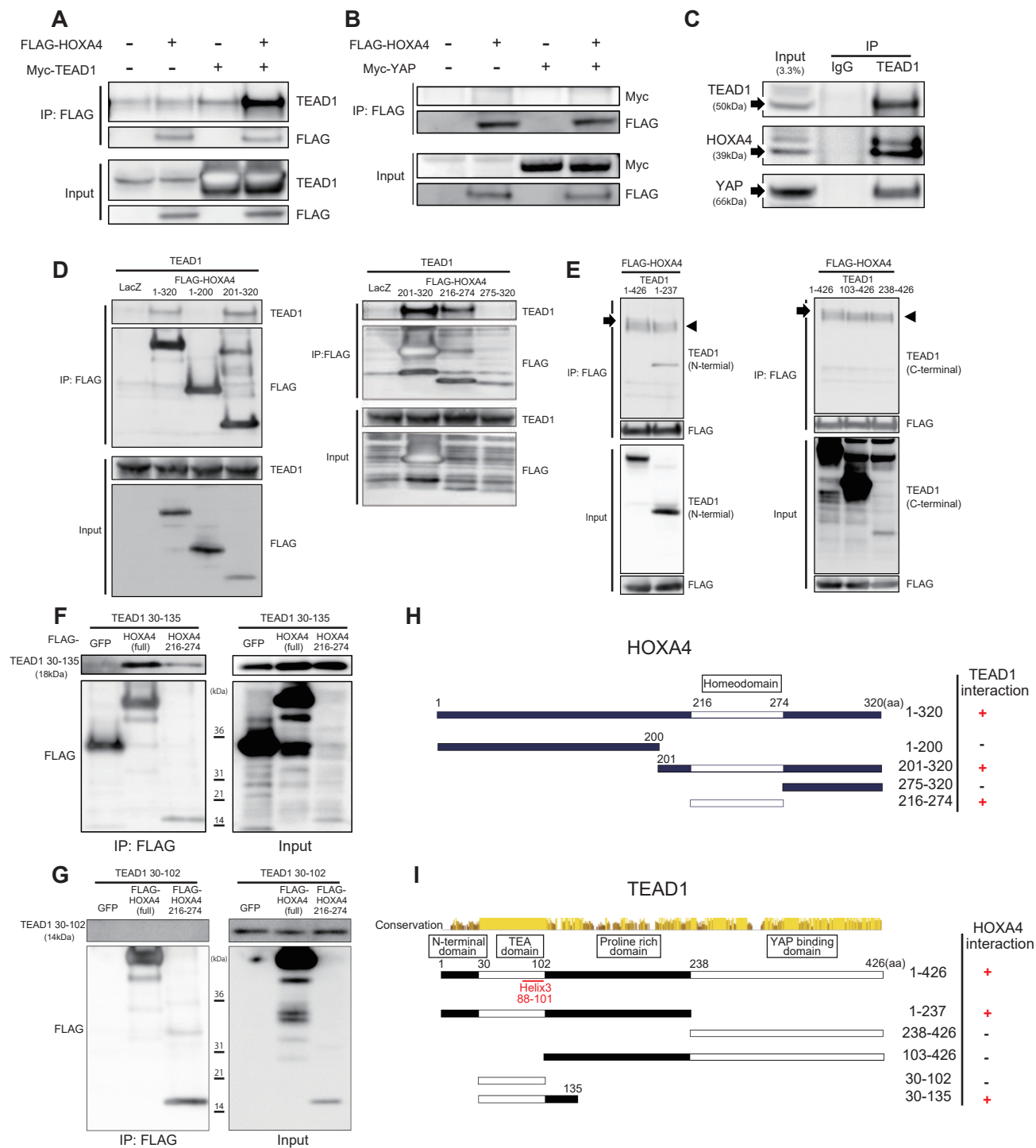


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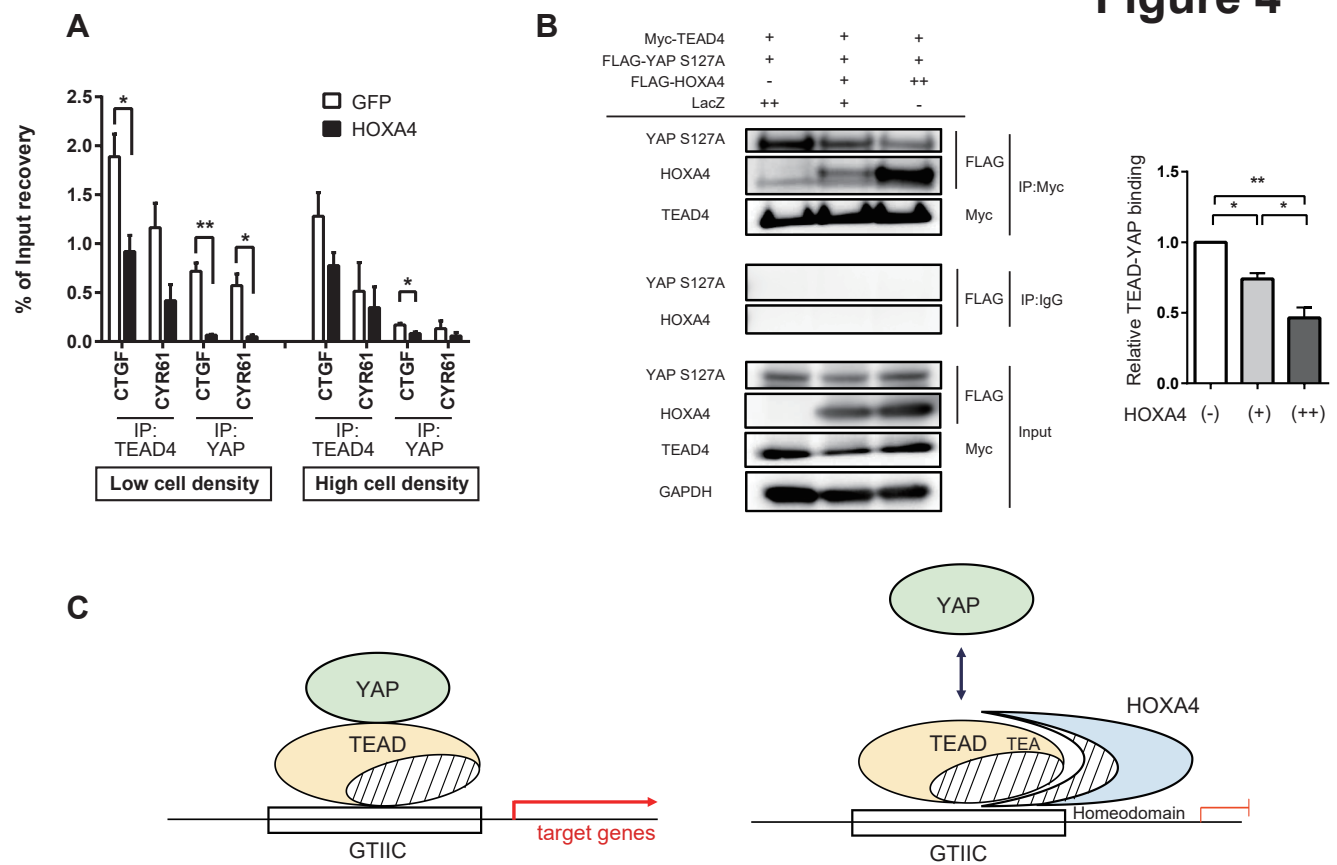


Figure 5

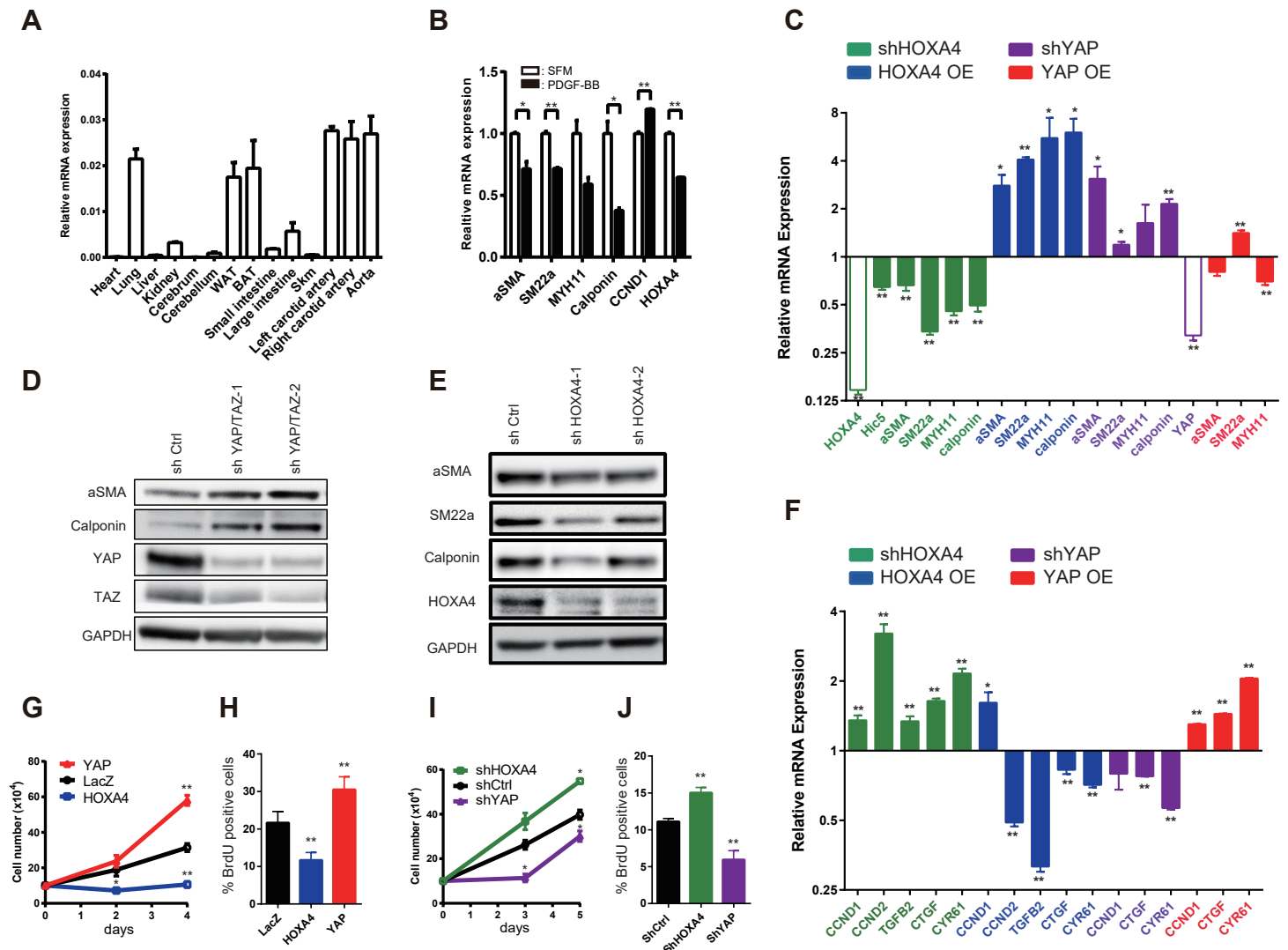
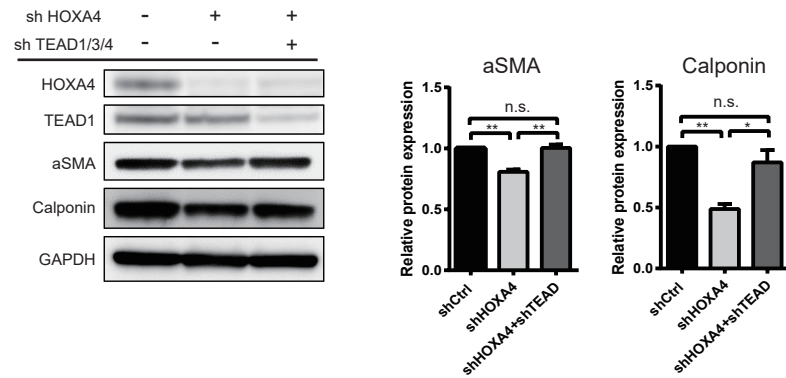
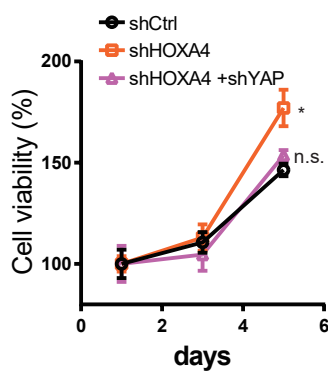


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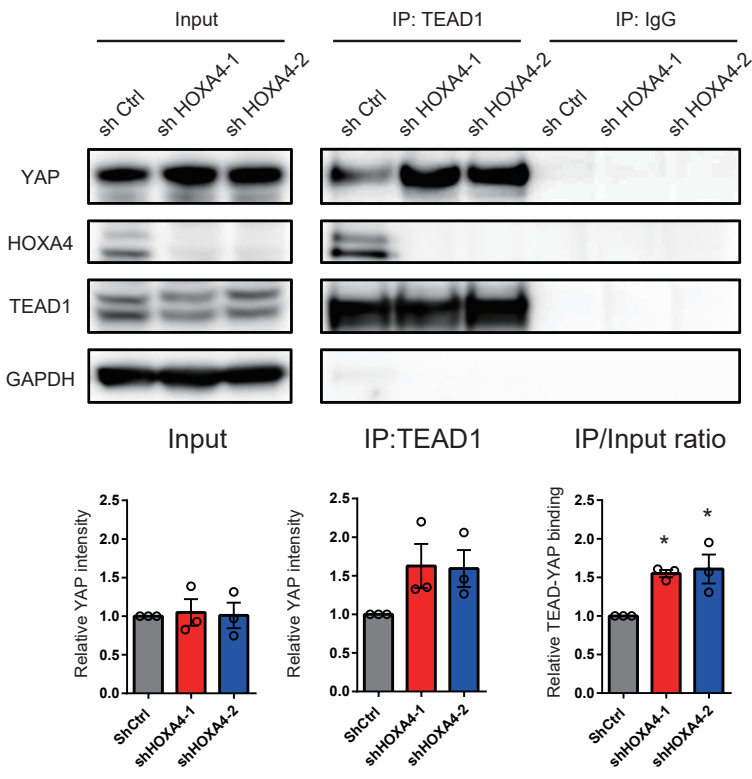
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B



C



D

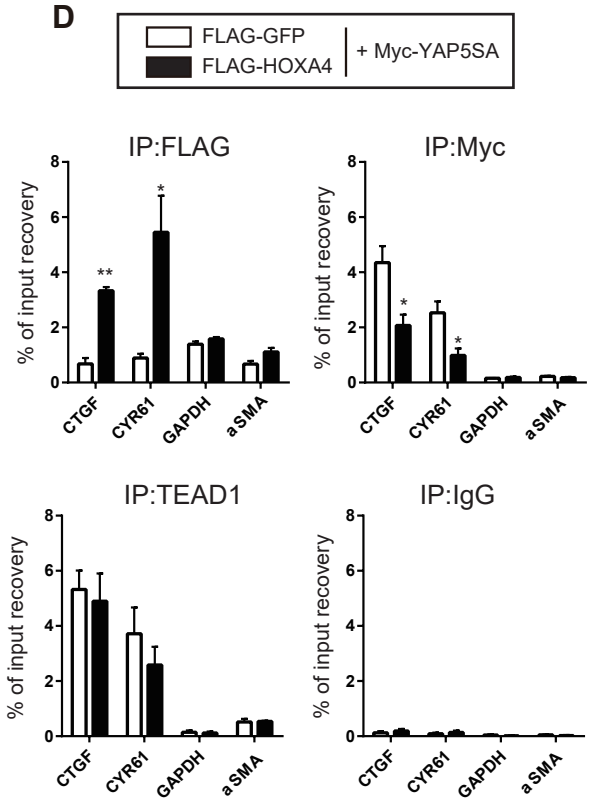
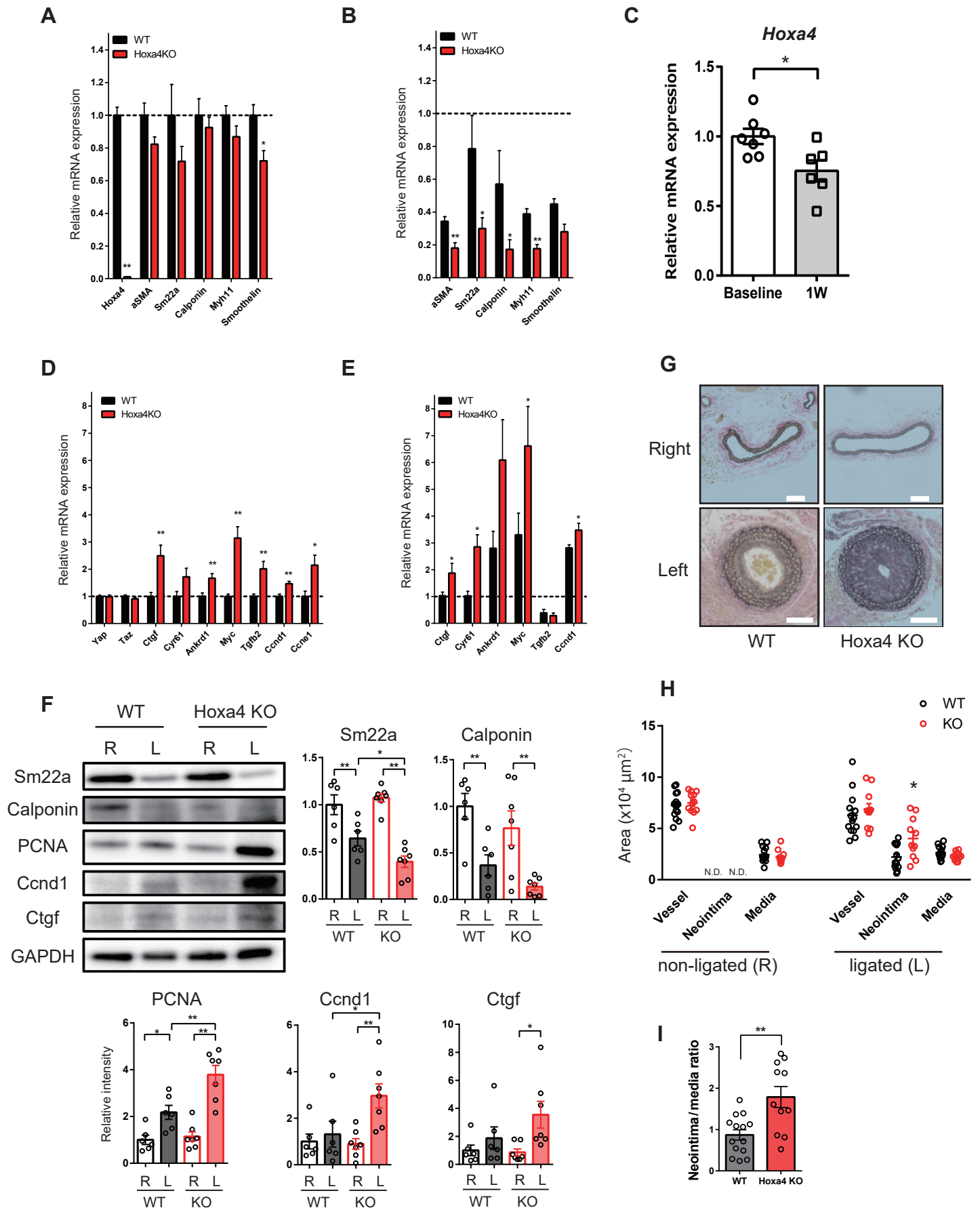
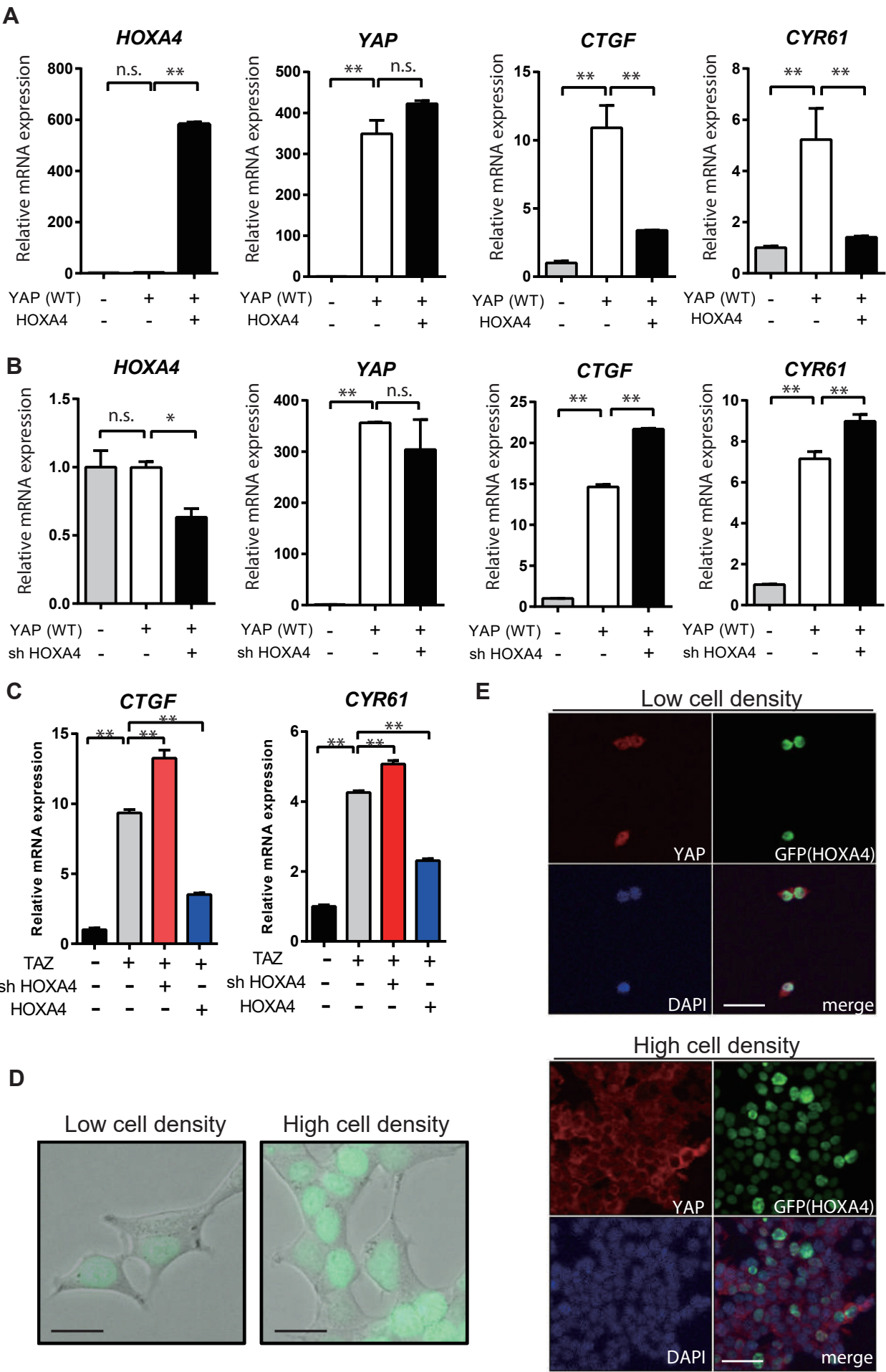


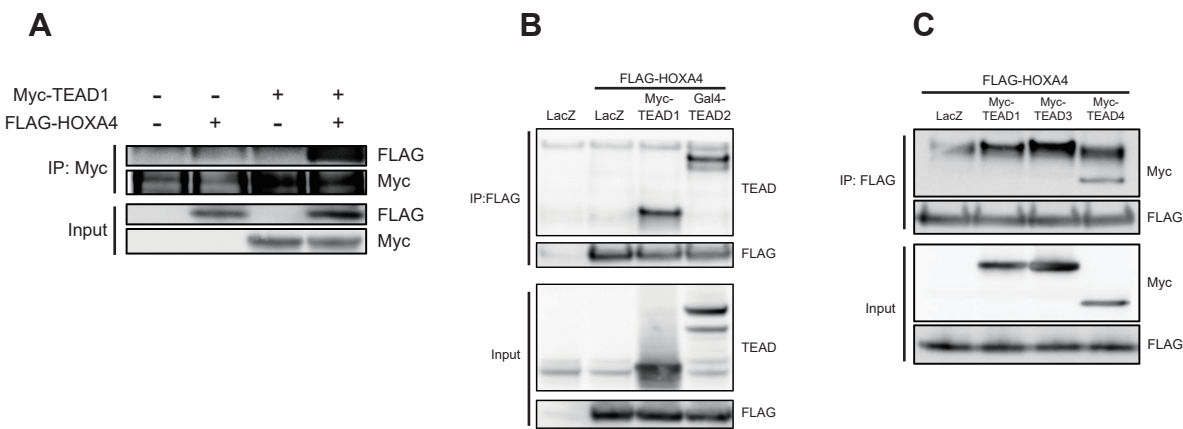
Figure 7



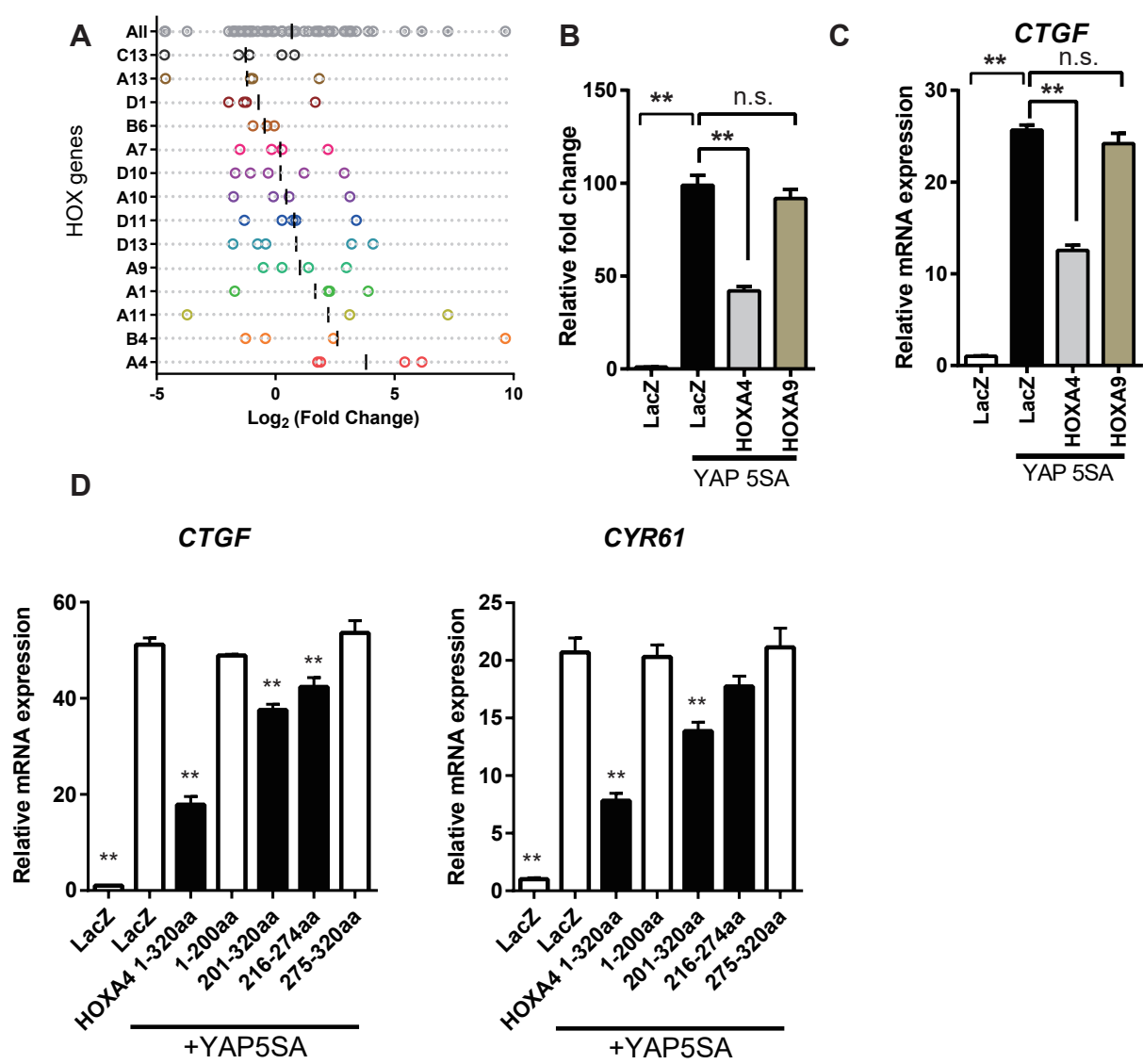
Expanded View Figure 1



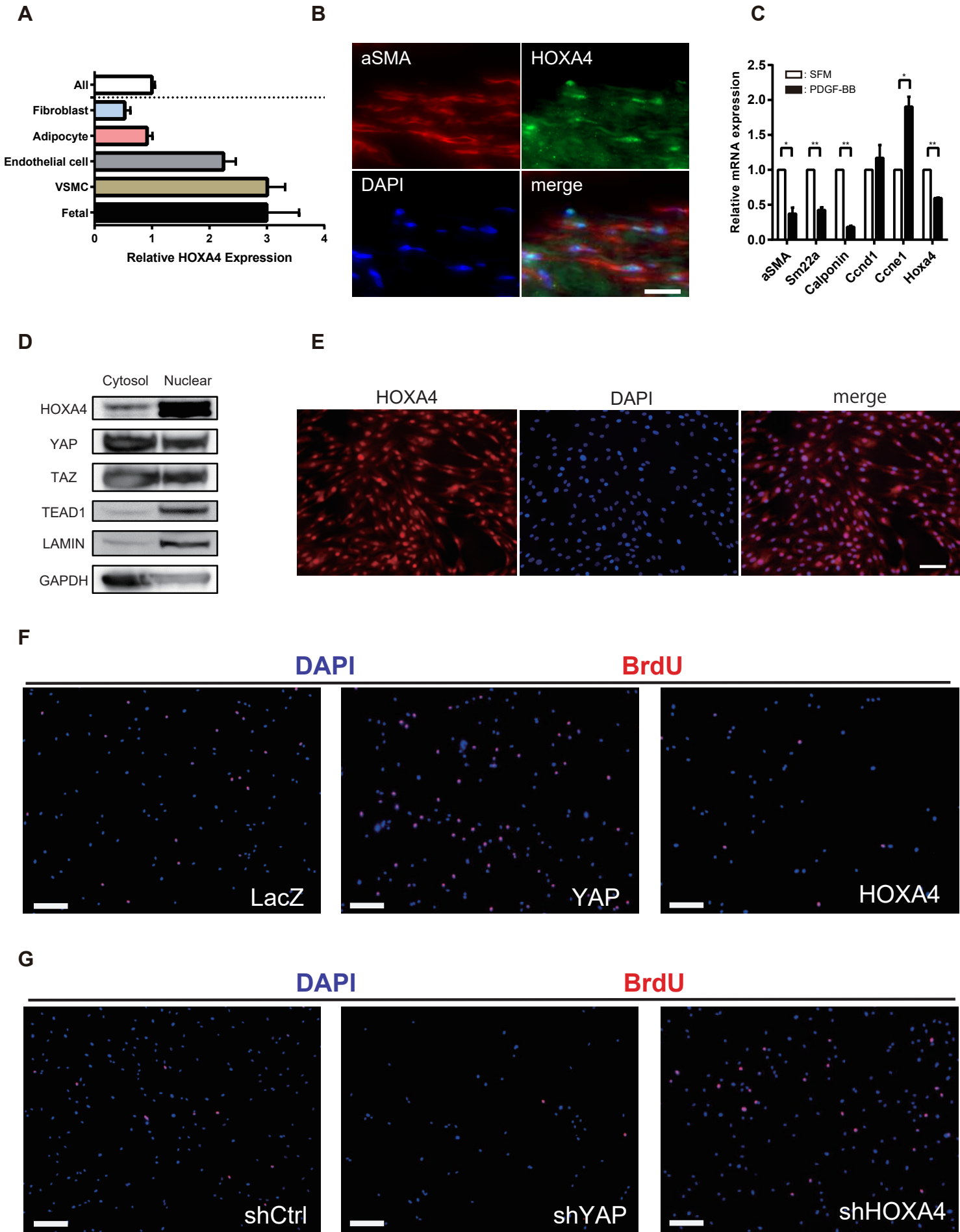
Expanded View Figure 2



Expanded View Figure 3

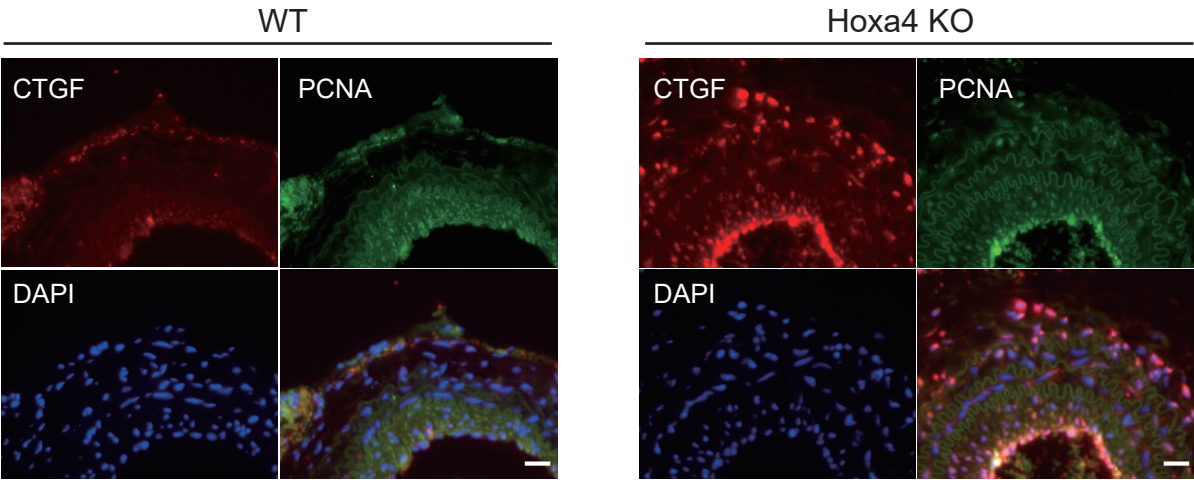


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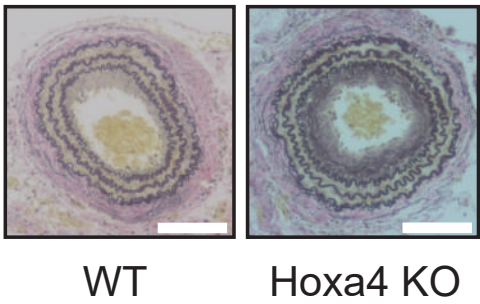


Expanded View Figure 5

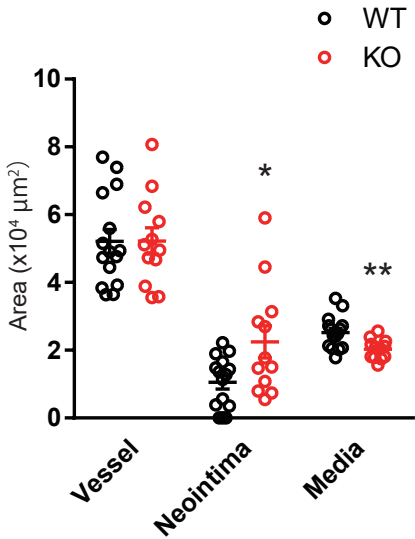
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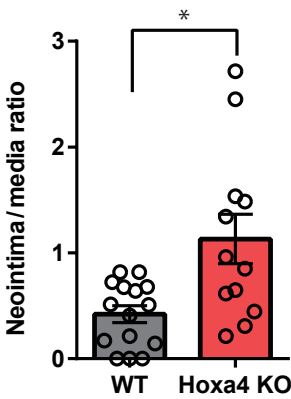
B



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D



Appendix for:

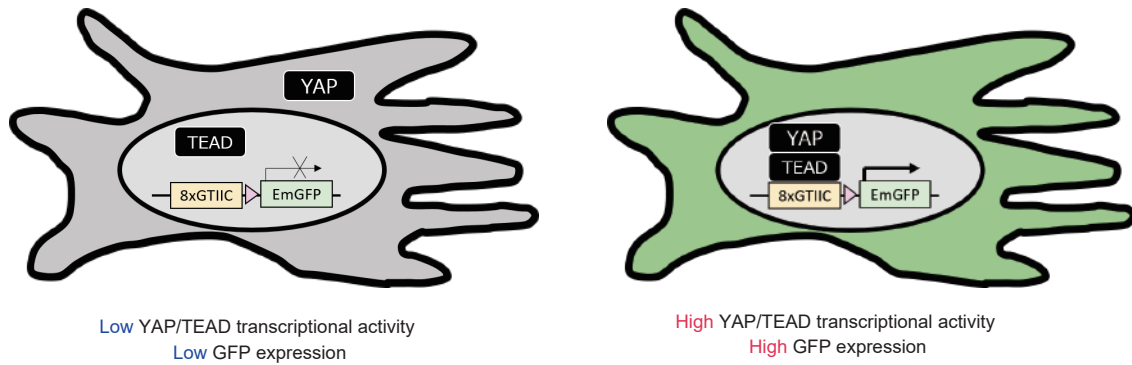
Homeobox A4 Suppresses Vascular Remodeling as a Novel Regulator of YAP/TEAD Transcriptional Activity

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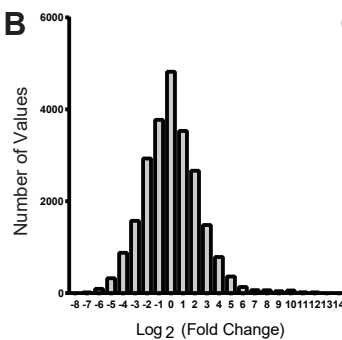
Table of contents

Appendix Figure S1 - Identification of novel modulators of YAP/TEAD transcriptional activity using pooled shRNA screen.....	2
Appendix Figure S2 - HOXA4 attenuated the expression of YAP/TEAD target genes.....	3
Appendix Figure S3 - Gene expression levels of canonical Hippo pathway kinases were unaffected by HOXA4.....	4
Appendix Figure S4 - Expression of TEAD family genes.....	5
Appendix Figure S5 - Generation of Hoxa4 deficient mice.....	6
Appendix Figure S6 – Characteristics of Hoxa4-deficient mice.....	7

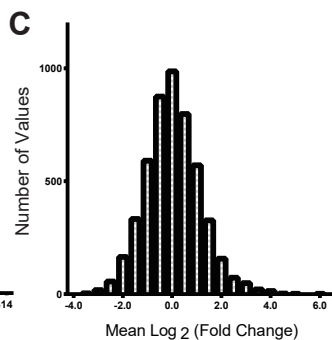
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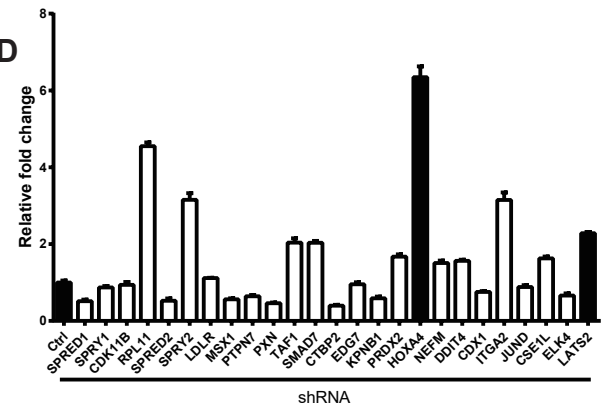
B



C



D

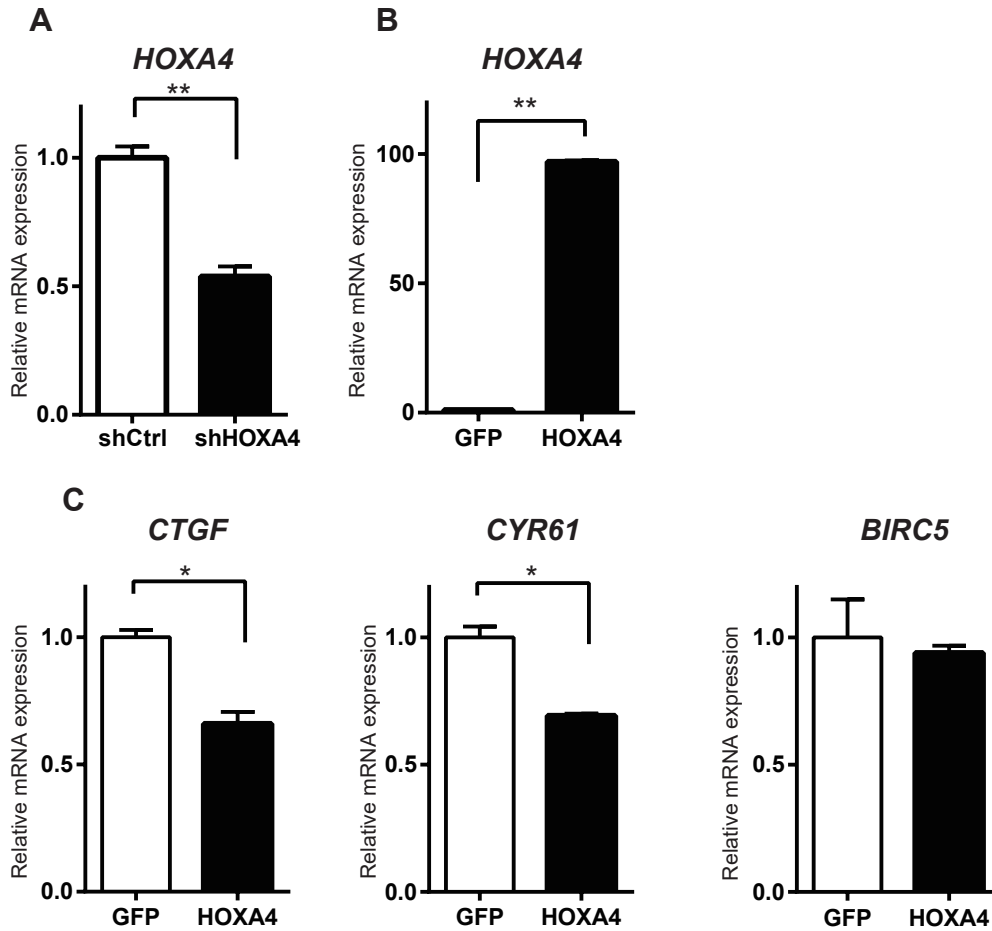


Appendix Figure S1 - Identification of novel modulators of YAP/TEAD transcriptional activity using pooled shRNA screen.

A, Schematic drawing of 8×GTIIC-EmGFP 293T cell line carrying eight TEAD-binding sequences upstream of a minimal promoter and EmGFP.

B and **C**, Distribution of read abundance per shRNA (**B**) or average read abundance of shRNA targeting each gene (**C**) followed an approximately Gaussian curve.

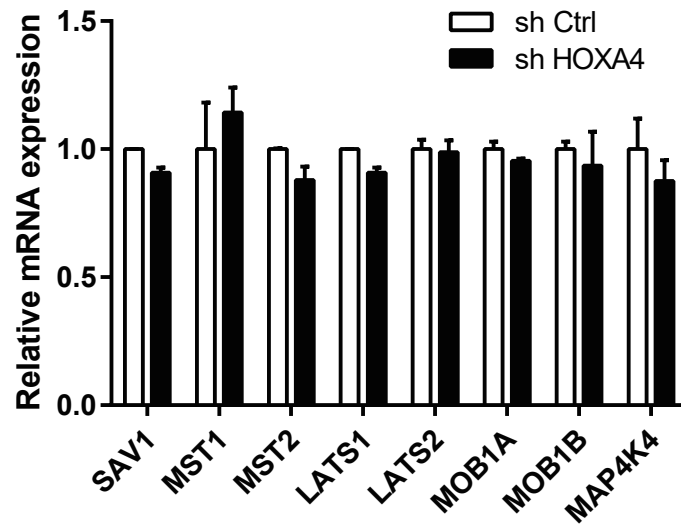
D, Validation of YAP/TEAD transactivation by knockdown of candidate genes using 8xGTIIC luciferase assay (n=3). Data are presented as mean ± SEM.



Appendix Figure S2 - HOXA4 attenuated the expression of YAP/TEAD target genes.

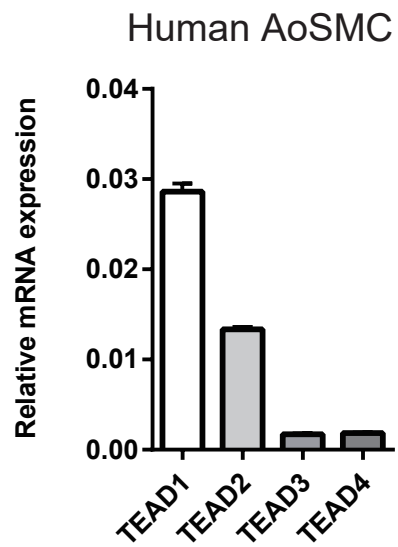
A, Knockdown efficacy of shHOXA4 vector in HEK 293T cells assessed by quantitative real-time PCR (n=3).

B and **C**, Quantitative real-time PCR analysis of HOXA4 (B) and YAP/TEAD target gene expression (C) in HEK 293T cells transfected with HOXA4 expression vector (n=3). All data are presented as mean $\pm$ SEM.



Appendix Figure S3 - Gene expression levels of canonical Hippo pathway kinases were unaffected by HOXA4.

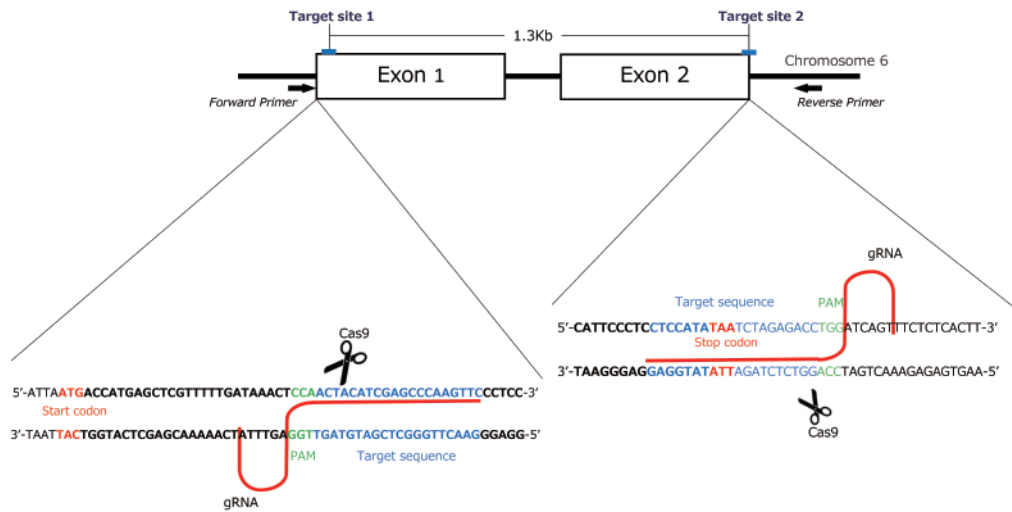
Quantitative real-time PCR analysis of core Hippo pathway kinases in HEK 293T cells transfected with shHOXA4 (n=3). Data are presented as mean $\pm$ SEM.



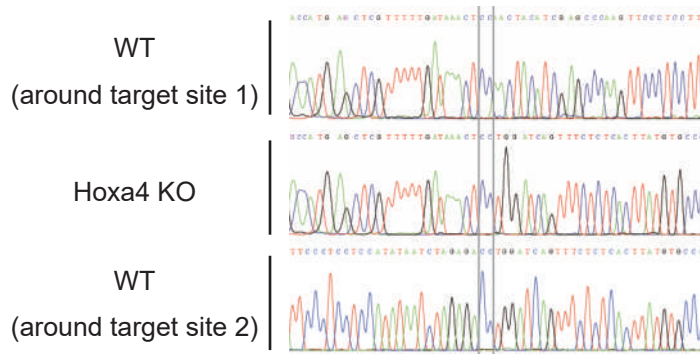
Appendix Figure S4 - Expression of TEAD family genes.

Quantitative real-time PCR analysis of TEAD family genes in human vascular smooth muscle cells (n=3). Data are presented as mean $\pm$ SEM.

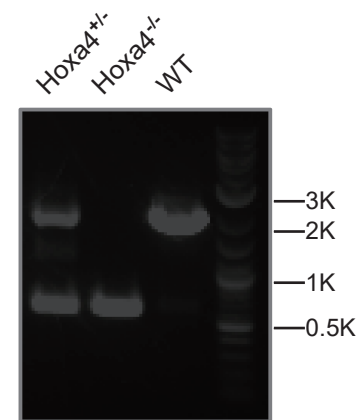
A



B



C

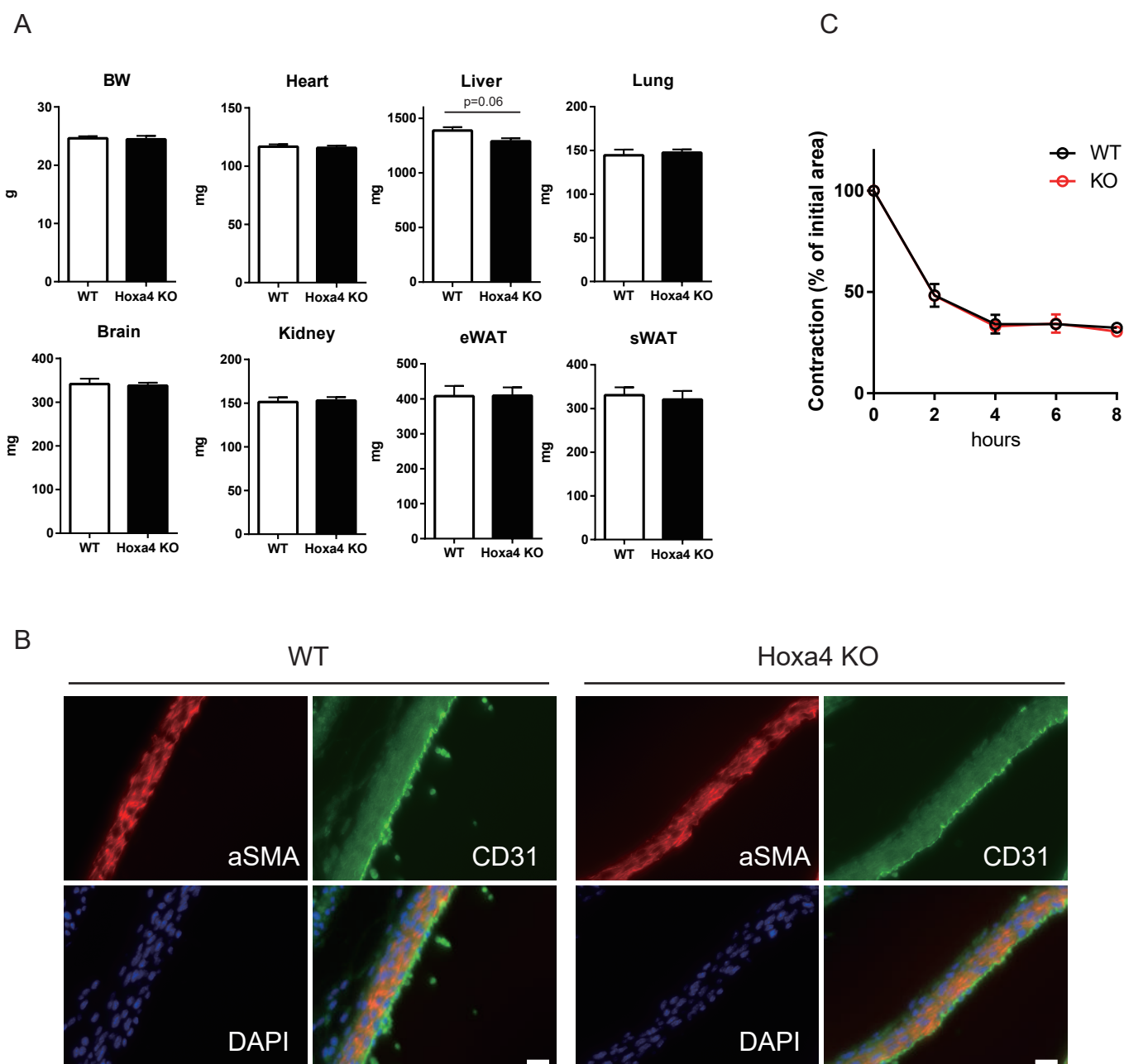


Appendix Figure S5 - Generation of Hoxa4 deficient mice.

A, Strategy to generate Hoxa4 KO mice.

B, DNA sequence around the target site of Cas9 cleavage in the knockout allele.

C, PCR analysis of mouse tail genome.



Appendix Figure S6 – Characteristics of Hoxa4-deficient mice.

A, Organ weight of 9-week-old male mice (WT: n=6, Hoxa4: n=8). Data are presented mean ± SEM and analyzed by unpaired two-tailed Mann–Whitney test. BW; body weight, eWAT; epididymal white adipose tissue, sWAT; subcutaneous white adipose tissue.

B, Representative immunofluorescent staining images for α-SMA and CD31 on dorsal aorta of embryonic day 18.5 (E18.5). Bars indicate 20 μm.

C, Collagen gel contraction assay of primary vascular smooth muscle cells harvested from 8-week-old male mice (n=3). Data are presented as mean ± SEM.