

1 **HIF1 $\alpha$ -AS1 is a DNA:DNA:RNA triplex-forming lncRNA interacting with the HUSH complex**

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49 The authors have declared that no conflict of interest exists.

50 Running Head: HIF1 $\alpha$ -AS1 and triplex formation

51 **Abstract**

52 DNA:DNA:RNA triplexes that are formed through Hoogsteen base-pairing have been observed *in*  
53 *vitro*, but the extent to which these interactions occur in cells and how they impact cellular functions  
54 remains elusive. Using a combination of bioinformatic techniques, RNA/DNA pulldown and  
55 biophysical studies, we set out to identify functionally important DNA:DNA:RNA triplex-forming long  
56 non-coding RNAs (lncRNA) in human endothelial cells. The lncRNA HIF1 $\alpha$ -AS1 was retrieved as a top  
57 hit. Endogenous HIF1 $\alpha$ -AS1 reduced the expression of numerous genes, including EPH Receptor A2  
58 and Adrenomedullin through DNA:DNA:RNA triplex formation by acting as an adapter for the  
59 repressive human silencing hub complex (HUSH). Moreover, the oxygen-sensitive HIF1 $\alpha$ -AS1 was  
60 down-regulated in pulmonary hypertension and loss-of-function approaches not only resulted in  
61 gene de-repression but also enhanced angiogenic capacity. As exemplified here with HIF1 $\alpha$ -AS1,  
62 DNA:DNA:RNA triplex formation is a functionally important mechanism of trans-acting gene  
63 expression control.

64 **Introduction**

65 Long non-coding RNAs (lncRNAs) represent the most diverse, plastic and poorly understood class of  
66 ncRNA<sup>1</sup>. Their gene regulatory mechanisms involve formation of RNA-protein, RNA-RNA or RNA-DNA  
67 complexes<sup>1</sup>. RNA-DNA interactions occur either in heteroduplex (DNA:RNA) or triplex strands  
68 (DNA:DNA:RNA). In triplexes, double-stranded DNA (dsDNA) accommodates the single-stranded RNA  
69 in its major groove<sup>2</sup>. The binding occurs via Hoogsteen or reverse Hoogsteen hydrogen bonds with a  
70 purine-rich sequence of DNA to which the RNA strand binds in a parallel or antiparallel manner.  
71 Hoogsteen bonds are weaker than Watson-Crick bonds, resulting in Hoogsteen pairing rules being  
72 more flexible<sup>3</sup>.

73 *Ex vivo* triplex formation relies on different biophysical methods including circular dichroism- (CD)  
74 and nuclear magnetic resonance-spectroscopy (NMR)<sup>4-6</sup>. Even with these techniques it can be  
75 challenging to discriminate DNA-RNA heteroduplexes from triplexes and analyses are usually  
76 restricted to oligonucleotides of a limited length. Nevertheless, a few lncRNAs have been suggested  
77 to form triplexes with dsDNA, however, triplex studies using living cells are still in early  
78 development<sup>4,6-13</sup>. *In silico* analyses of RNA-DNA triplex formation predicted several genomic loci and  
79 lncRNAs to form triplexes<sup>14</sup>. In line with this, a global approach in HeLa S3 and U2OS cells to isolate  
80 triplex-forming RNAs on a genome-wide scale yielded several RNA:DNA triplex-forming lncRNAs<sup>15</sup>.

81 In addition to the sparse initial findings of triplex formation within cells, several other open questions  
82 remain: What is the physiological relevance of triplex-forming lncRNAs and are these cell- and tissue-  
83 type specific? What is the mechanism of action of triplex-forming lncRNAs? Do they disturb  
84 transcription in a similar way to R-loops<sup>16</sup> or recruit certain protein complexes to DNA in a site-  
85 specific manner? Regarding the latter aspect, Polycomb Repressive Complex 2 (PRC2) has been  
86 identified as a target of the lncRNAs HOX Transcript Antisense RNA (HOTAIR), FOXF1 Adjacent Non-  
87 Coding Developmental Regulatory RNA (FENDRR) and Maternally Expressed 3 (MEG3)<sup>4,12,13</sup>, but, given  
88 the highly promiscuous nature of PRC2, this function remains controversial. Other examples of  
89 protein interactors involve e.g. E2F1 and p300, which are recruited by the triplex-forming antisense  
90 lncRNA KHPS1 to activate gene expression of the proto-oncogene sphingosine kinase 1 (SPHK1) *in*  
91 *cis*<sup>7,10</sup>.

92 Much of today's *in vivo* RNA research heavily relies on immortalized cell lines. Although such model  
93 systems are well suited for transfection or genomic manipulation, they are highly de-differentiated  
94 and exhibit reaction patterns such as unlimited growth and immortalization - characteristics not  
95 observed in primary cells<sup>17</sup>. Considering that lncRNAs are expressed in a species-, tissue- and  
96 differentiation-specific manner<sup>1</sup>, biological evidence for lncRNA functions in primary cells is limited.  
97 Among such cells, endothelial cells stand out due to their well documented importance in

98 regeneration, angiogenesis and tissue vascularization. Indeed, endothelial cell dysfunction is one of  
99 the main drivers of systemic diseases like diabetes and inflammation<sup>18</sup>.

100 Here, we combined molecular biology and biophysics, bioinformatics and physiology to  
101 systematically uncover the role of triplex-forming lncRNAs in endothelial cells. This approach  
102 identified HIF1 $\alpha$ -AS1 as a *trans*-acting triplex-forming lncRNA that controls vascular gene expression  
103 in endothelial cells with implications for vascular disease.

104 **Results**

105 *HIF1 $\alpha$ -AS1 is a triplex-associated lncRNA*

106 To identify triplex-associated lncRNAs, we used Triplex-Seq data from U2OS and HeLa S3 cells<sup>15</sup>.  
107 Triplex-Seq relies on the isolation of RNase H-resistant RNA-DNA complexes from cells followed by  
108 DNA- and RNA-Seq<sup>15</sup>. The data comprised all RNA entities and was filtered for lncRNAs, resulting in  
109 989 (for HeLa S3, **Sup. Table 1**) and 1386 (for U2OS, **Sup. Table 2**) lncRNA regions associated with  
110 triplexes, with an overlap of 280 regions between the two cell lines (**Fig. 1a**). To further narrow down  
111 this set of enriched triplex-associated lncRNAs, parameters for specificity (fold enrichment >10,  
112 minus\_log10(P) >20) were increased so that 11 lncRNA candidates with high confidence remained.  
113 Subsequently, these were correlated to Encode and FANTOM5 Cap Analysis of Gene Expression  
114 (CAGE)<sup>19–21</sup> data. Of the 11 candidates, only 5 (RMRP, HIF1 $\alpha$ -AS1, RP5-857K21.4, SCARNA2 and  
115 SNHG8) were expressed in endothelial cells. All 5 candidates were predicted as non-coding by the  
116 online tools Coding Potential Assessment Tool (CPAT) and coding potential calculator 2 (CPC2) and at  
117 least partially nuclear localized by Encode and FANTOM5 CAGE (**Fig. 1a**). To further analyze these  
118 candidates, the Triplex-Seq enriched regions were manually inspected in the IGV browser. This led to  
119 the exclusion of SNHG8 as the triplex-associated regions within this lncRNA were exclusively within  
120 the overlapping small nucleolar RNA 24 (SNORA24) gene. In the case of the other candidates, triplex-  
121 association was within the individual lncRNA gene body. The cumulative fold enrichment of the  
122 remaining lncRNAs in the Triplex-Seq dataset illustrated strong triplex-association (**Extended data**  
123 **Fig. 1a**). To verify the candidates experimentally, RNA immunoprecipitation (RIP) with antibodies  
124 against dsDNA and with or without RNase H treatment in human endothelial cells was performed.  
125 RNase H, which cleaves the RNA in DNA-RNA heteroduplexes (R-loops)<sup>22</sup>, revealed that HIF1 $\alpha$ -AS1  
126 was the strongest triplex-associated lncRNA (**Fig. 1b**).

127 Genomically, HIF1 $\alpha$ -AS1 is located on the antisense strand of the Hypoxia-inducible factor 1 $\alpha$  gene  
128 (**Fig. 1c**). The lncRNA was specifically enriched in nuclear DNA, whereas HIF1 $\alpha$  mRNA and 18S rRNA  
129 were not (**Fig. 1d**). Moreover, RIP with anti-histone 3 (**Fig. 1e**) indicated that HIF1 $\alpha$ -AS1 is bound to  
130 dsDNA in the chromatin environment.

131 *HIF1 $\alpha$ -AS1 is disease-relevant*

132 Only a few studies have so far reported the biological relevance of HIF1 $\alpha$ -AS1. Increased HIF1 $\alpha$ -AS1  
133 expression has been reported in thoracoabdominal aortic aneurysms<sup>23</sup>. HIF1 $\alpha$ -AS1 was also  
134 suggested as a biomarker in colorectal carcinoma<sup>24</sup>. Functionally, HIF1 $\alpha$ -AS1 is pro-apoptotic and  
135 anti-proliferative in vascular smooth muscle, Kupffer and umbilical vein endothelial cells<sup>25–27</sup>.

136 As HIF1 $\alpha$  is a central regulator of oxygen-dependent gene expression<sup>18</sup>, we decided to measure the  
137 expression of HIF1 $\alpha$ -AS1 in endothelial cells in altered oxygen and disease conditions. Hypoxia led to  
138 a decrease in HIF1 $\alpha$ -AS1 expression in endothelial and pulmonary artery smooth muscle cells  
139 (paSMC) (**Fig. 1f, Extended data Fig. 1b**), which was restored in endothelial cells after 4 h and even  
140 surpassed basal levels after 24 h of normoxic conditions (**Fig. 1g**). Importantly, HIF1 $\alpha$ -AS1 was  
141 downregulated in endothelial cells isolated from human glioblastoma (**Extended data Fig. 1c**) and in  
142 lungs from patients with end stage idiopathic pulmonary arterial hypertension (IPAH) or chronic  
143 thromboembolic pulmonary hypertension (CTEPH) (**Fig. 1h**). In paSMCs isolated from pulmonary  
144 arteries of patients with IPAH, HIF1 $\alpha$ -AS1 was strongly decreased (**Extended data Fig. 1d**). Together,  
145 these data demonstrate that HIF1 $\alpha$ -AS1 is an oxygen-dependent and disease-relevant lncRNA.

#### 146 *HIF1 $\alpha$ -AS1-triplex binding suppresses target gene expression*

147 Triplex-Seq provides evidence for existing triplex forming regions of the RNA (TFR) and triplex target  
148 sites (TTS) within the DNA but the details of exactly which TFR and TTS interact cannot be derived  
149 from Triplex-Seq. To identify the TFRs within HIF1 $\alpha$ -AS1 as well as HIF1 $\alpha$ -AS1-dependent TTS, a  
150 combination of bioinformatics and wet lab approaches were used: An Assay for Transposase-  
151 Accessible Chromatin with high-throughput sequencing (ATAC-Seq) was performed after HIF1 $\alpha$ -AS1  
152 knockdown to identify DNA target sites in human endothelial cells. LNA-GapmeRs targeting HIF1 $\alpha$ -  
153 AS1 led to a strong knockdown of the lncRNA (**Extended data Fig. 1e**). Triplex Domain Finder (TDF)  
154 predicted the TFRs within HIF1 $\alpha$ -AS1 to target DNA regions around genes that displayed altered  
155 ATAC-Seq peaks after HIF1 $\alpha$ -AS1 silencing (**Fig. 2a**). The software identified three statistically  
156 significant TFRs (TFR1-3) within the pre-processed HIF1 $\alpha$ -AS1 RNA (**Fig. 2b**). There was also a high  
157 incidence of triplex-prone motifs predicted in regions whose chromatin state was altered in the  
158 ATAC-Seq data after HIF1 $\alpha$ -AS1 knockdown (**Fig. 2c, Sup. Tables 3-5**). Of these TTS, 38 overlapped  
159 within all three TFRs (**Fig. 2d**). To identify which TFR is most strongly associated with triplexes, RIP  
160 with S9.6 antibodies recognizing RNA-DNA association was performed. RNA-DNA associations  
161 remaining after RNase H treatment excluded the possibility that these were RNA-DNA  
162 heteroduplexes. Of the three HIF1 $\alpha$ -AS1 TFRs, TFR2 was identified as the TFR most resistant to RNase  
163 H (**Fig. 2e**). TFR2 is located intronically 478 nucleotides (nt) downstream of Exon1 and was detected  
164 by RT-PCR within nuclear isolated RNA with primers covering the first 714 nt (E1-I) of the pre-  
165 processed HIF1 $\alpha$ -AS1 (**Extended data Fig. 1f**). Triplex-prone motifs in their target regions yielded  
166 more than 20 different associated genes, some of which displayed a high number of DNA binding  
167 sites (**Fig. 2f**). If this binding of the lncRNA is truly relevant for the individual target gene, then a  
168 change in target gene expression would be expected. Importantly, in response to the downregulation  
169 of HIF1 $\alpha$ -AS1 with LNA-GapmeRs the expression of the following triplex target genes increased:

170 ADM, PLEC, RP11-276H7.2, EPHA2, MIDN and EGR1 (**Fig. 2g**). Interestingly, as exemplified by the  
171 target genes HIF1 $\alpha$ , EPHA2 and ADM, the triplex target sites are often located close to the 5' end of  
172 the gene. In this region histone modifications, transcription factor binding and chromatin  
173 conformation often have the greatest effect on promoter function and gene expression (**Fig. 2h**).

174 These data indicate that HIF1 $\alpha$ -AS1 contains triplex forming regions and target sites important for  
175 the regulation of gene expression.

#### 176 *HIF1 $\alpha$ -AS1 TFR2 RNA forms triplexes with EPHA2 and ADM*

177 Our analysis identified HIF1 $\alpha$ -AS1 TFR2 as the best suited candidate for verification of triplex  
178 formation of the lncRNA using biophysical and biochemical techniques. To monitor triplex formation  
179 of HIF1 $\alpha$ -AS1, EPHA2 was chosen as the target gene due to its abundance of triplex target sites (**Fig.**  
180 **2f, Fig. 2h**), its regulatory potential (**Fig. 2g**) and its importance for vascularization<sup>28</sup>. The formation of  
181 DNA:DNA:RNA triplexes between lncRNA HIF1 $\alpha$ -AS1 TFR2 and its proposed DNA target site within  
182 intron 1 of EPHA2 was characterized by solution NMR spectroscopy, electrophoretic mobility shift  
183 assay (EMSA) and CD-spectroscopy. <sup>1</sup>H-1D NMR spectra were recorded for EPHA2 DNA duplex,  
184 HIF1 $\alpha$ -AS1 TFR2 RNA (TFO2-23), EPHA2:HIF1 $\alpha$ -AS1\_TFR2 heteroduplex and EPHA2:HIF1 $\alpha$ -AS1\_TFR2  
185 triplex at different temperatures. Using 10 eq HIF1 $\alpha$ -AS1 TFR2 RNA, triplex <sup>1</sup>H NMR imino signals  
186 were observed in a spectral region between 9 and 12 ppm providing further evidence that HIF1 $\alpha$ -AS1  
187 was associated with EPHA2 through Hoogsteen base pairing (**Fig. 3a**). Moreover, HIF1 $\alpha$ -AS1 TFR2  
188 RNA formed a low mobility DNA–RNA complex with the radiolabeled EPHA2 DNA target sequence in  
189 electrophoretic mobility shift assays (EMSA). The shift in mobility retardation was dependent on the  
190 TFR2 transcript length (**Fig. 3b**). We also used CD-spectroscopy to confirm triplex formation of HIF1 $\alpha$ -  
191 AS1 TFR2 on EPHA2. The CD spectrum indicated typical features for triplex formation, such as a  
192 positive small peak at ~220 nm, two negative peaks at ~210 nm and ~240 nm and a blue-shift of the  
193 peak at ~270 nm, which was distinct from the EPHA2 DNA duplex or the heteroduplex spectra (**Fig.**  
194 **3c**). This confirmed the existence of EPHA2:HIF1 $\alpha$ -AS1 TFR2 triplexes. Additionally, we performed UV  
195 melting assays and obtained melting temperatures  $T_m$  (RNA-DNA heteroduplex) =  $53.48 \pm 0.32$  °C,  $T_m$   
196 (DNA-DNA duplex) =  $70.73 \pm 0.22$  °C and  $T_m$  (DNA-DNA-RNA triplex) =  $54.17 \pm 0.23$  °C with a very  
197 broad second melting point around 70 °C. The biphasic melting transition is a distinct feature of  
198 triplex formation, where the first melting temperature corresponds to melting of Hoogsteen  
199 hydrogen bonds that stabilize the triplex and the second for the melting of the Watson-Crick base  
200 pairing at higher temperatures (**Fig. 3d**).

201 To confirm the formation of triplexes with lower equivalents, stabilized triplex formation was  
202 investigated: the intermolecular dsDNA form from two complementary antiparallel DNA strands was



203 changed into a hairpin construct, where both DNA strands were linked with a 5 nt thymidine-linker  
204 and duplex formation thus became intramolecular. With this approach, triplex formation was  
205 obtained with 3 eq RNA, indicating that triplex formation is favored under those conditions as  
206 expected. <sup>1</sup>H-1D NMR spectra of hairpin EPHA2\_CTGA and 15N HIF1 $\alpha$ -AS1 TFR2:EPHA\_CTGA triplex  
207 indicated changes in the Hoogsteen region (9-12 ppm) and the spectral region of imino (12-14 ppm)  
208 and amino signals (7-8.5 ppm) (**Extended data Fig. 2a**). In addition to EPHA2, we also tested ADM, a  
209 preprohormone involved in endothelial cell function<sup>29</sup>. For ADM\_CTGA:HIF1 $\alpha$ -AS1 TFR2 triplex, the  
210 new imino protons in the Hoogsteen region arose at lower temperatures (**Extended data Fig. 2b**). For  
211 both ADM\_CTGA and EPHA2\_CTGA triplex constructs the CD spectra showed an increased negative  
212 ellipticity at ~240 nm and positive ellipticity at ~270 nm (**Extended data Fig. 2c,e**). Further, the UV  
213 melting data verified the triplex stabilization with higher melting temperatures and defined melting  
214 transitions upon DNA hairpin formation. For the EPHA2\_CTGA:HIF1 $\alpha$ -AS1 TFR2 (TFO2-23) triplex we  
215 obtained a first melting point at  $T_m$  (1<sup>st</sup> triplex) = 50.08  $\pm$  0.51 °C, a second melting point  $T_m$  (2<sup>nd</sup>  
216 triplex) = 79.90  $\pm$  0.10 °C and  $T_m$  (DNA hairpin) = 80.41  $\pm$  0.10 °C (**Extended data Fig. 2d**). The melting  
217 temperature of ADM DNA duplex  $T_m$  (DNA-DNA duplex) = 63.80  $\pm$  0.20 °C increased for the  
218 ADM\_CTGA hairpin  $T_m$  (DNA hairpin) = 95.76  $\pm$  16.69 °C. For the ADM\_CTGA:HIF1 $\alpha$ -AS1 TFR2 (TFO2-  
219 23), we obtained a first melting point  $T_m$  (1st triplex) = 51.19  $\pm$  0.68 °C and a second  $T_m$  (2nd triplex) =  
220 82.86  $\pm$  0.21 °C (**Extended data Fig. 2f**). The data demonstrate that HIF1 $\alpha$ -AS1 TFR2 forms triplexes  
221 with EPHA2 and ADM dsDNA under regular and triplex-stabilized conditions upon DNA hairpin  
222 formation.

### 223 *TFR2 represses EPHA2 and ADM gene expression*

224 The current data indicates that HIF1 $\alpha$ -AS1 forms triplexes with EPHA2 and ADM, however, the  
225 mechanistic and functional consequences of this phenomenon are unclear. To investigate these  
226 aspects, gain and loss of function approaches were performed. Increasing the expression of HIF1 $\alpha$ -  
227 AS1 using a dCas9-VP64 CRISPR activation system (CRISPRa) reduced the expression of EPHA2 and  
228 ADM (**Fig. 4a**). Conversely, downregulation of HIF1 $\alpha$ -AS1 with a dCas9-KRAB repression system  
229 (CRISPRi) increased the expression of EPHA2 and ADM (**Fig. 4b**). Consistent with HIF1 $\alpha$ -AS1  
230 repressing EPHA2 and ADM gene expression, EPHA2 levels increased after knockdown of HIF1 $\alpha$ -AS1  
231 (**Fig. 2g, Fig. 4c**). EPHA2 has a multi-faceted role in angiogenesis<sup>28,30,31</sup>. In HUVEC, knockdown of  
232 EPHA2 with siRNAs strongly reduced its RNA and protein expression and inhibited angiogenic  
233 sprouting (**Fig. 4d&e, Extended data Fig. 3a-c**). Conversely, a knockdown of HIF1 $\alpha$ -AS1 with LNA-  
234 GapmeRs increased basal, VEGF-A- and bFGF-mediated angiogenic sprouting (**Fig. 4f-g, Extended**  
235 **data Fig. 3d**), confirming the repressive effect of HIF1 $\alpha$ -AS1 on EPHA2. To demonstrate directly that  
236 TFR2 is responsible for the regulation of EPHA2, we replaced TFR2 by genome editing using a

237 recombinant Cas9-eGFP, a gRNA targeting TFR2 and different single-stranded oligodeoxynucleotides  
238 (ssODN) harboring either the published MEG3 TFR<sup>4</sup> or a luciferase control sequence (**Fig. 4h**).  
239 Replacement of the TFR2 with the MEG3 TFR, which served as a positive control for a functional TFR  
240 repressing TGFBR1 expression<sup>4</sup>, yielded a reduction in TGFBR1 levels compared to the luciferase  
241 control (**Fig. 4i**). More importantly, the loss of TFR2 consequently led to a loss of HIF1 $\alpha$ -AS1 TFR2, an  
242 upregulation of EPHA2 and partially of ADM (**Fig. 4j&k, Extended data Fig.3e**). These data  
243 demonstrate that TFR2 represses EPHA2 and ADM gene expression.

#### 244 *HIF1 $\alpha$ -AS1 binds to and recruits HUSH to triplex targets*

245 To elucidate the mechanism by which HIF1 $\alpha$ -AS1 represses gene expression, HIF1 $\alpha$ -AS1-associated  
246 proteins were studied using RNA pulldown experiments. 3'biotinylated spliced HIF1 $\alpha$ -AS1 lncRNA or  
247 3'biotinylated pcDNA3.1+ negative control were incubated in nuclear extracts from HUVECs and  
248 RNA-associated proteins were identified by electrospray ionization mass spectrometry, which  
249 retrieved M-phase phosphoprotein 8 (MPP8)-a component of the human silencing hub (HUSH)  
250 complex- as top hit (**Fig. 5a-b, Sup. Table 6**). The HUSH-complex is a nuclear machinery originally  
251 thought to mediate gene silencing during viral infection by recruiting the SET Domain Bifurcated  
252 Histone Lysine Methyltransferase 1 (SETDB1) which methylates H3K9<sup>32</sup>. The HUSH complex has not  
253 yet been studied in vascular cells and an interaction of its core protein MPP8 with lncRNAs has not  
254 been reported. To support our finding, RIP revealed that HIF1 $\alpha$ -AS1 and its TFR2, but not HIF1 $\alpha$   
255 mRNA, interact with MPP8 (**Fig. 5c, Extended data Fig. 4a-b**). Furthermore, HIF1 $\alpha$ -AS1 was highly  
256 enriched with H3K9me3 (**Fig. 5d**).

257 To map the RNA binding region of MPP8 on HIF1 $\alpha$ -AS1, we used *cat*RAPID fragments<sup>33</sup>, an algorithm  
258 involving division of polypeptide and nucleotide sequences into fragments to estimate the  
259 interaction propensity of protein-RNA pairs. This highlighted potential binding regions within Exon1  
260 (**Extended data 4c**). To substantiate these data experimentally, *ex vivo* bindings assays were  
261 performed between fragments of HIF1 $\alpha$ -AS1 and recombinant MPP8 (**Fig. 5e**). MPP8 interacted  
262 directly with HIF1 $\alpha$ -AS1 full length and a HIF1 $\alpha$ -AS1 mutant lacking Exon2 (**Fig. 5f**). In contrast and in  
263 accordance with the *cat*RAPID prediction, deletion of Exon1 (nucleotides 26-78nt in particular)  
264 prevented the interaction (**Fig. 5f**), indicating that this region of HIF1 $\alpha$ -AS1 is critical for the  
265 interaction of HIF1 $\alpha$ -AS1 with MPP8.

266 To demonstrate that HIF1 $\alpha$ -AS1 acts through HUSH complex recruitment, we first tested whether  
267 this complex exists in endothelial cells. Proximity ligation assays with antibodies against MPP8,  
268 dsDNA, H3K9me3 and SETDB1 confirmed the association of MPP8 with dsDNA (**Extended data Fig.**

269 **4d**), H3K9me3 (**Fig. 5g**) and SETDB1 (**Fig. 5h**) in the nuclei of endothelial cells, indicating that the  
270 complex is present in endothelial chromatin.

271 Chromatin immunoprecipitation (ChIP) with and without RNase A revealed that targeting of MPP8 to  
272 the HIF1 $\alpha$ -AS1 TTS of EPHA2 and ADM was attenuated after RNA depletion (**Fig. 6a**). To demonstrate  
273 the dependence of the interactions with the TTS on HIF1 $\alpha$ -AS1, ChIP experiments with antibodies  
274 targeting SETDB1, MPP8 and NP220 with or without knockdown of HIF1 $\alpha$ -AS1 were performed.  
275 NP220 (ZNF638), which is another member of the HUSH complex, interacted with HIF1 $\alpha$ -AS1, albeit  
276 to a lower degree than MPP8 (**Fig. 5b**). The binding of SETDB1 and MPP8, but not of NP220, to the  
277 triplex target sites of HIF1 $\alpha$ -AS1 required the presence of the lncRNA (**Fig. 6b-c**) suggesting that these  
278 interactions facilitate epigenetic processes and ultimately regulate gene expression. ATAC-Seq  
279 confirmed that these factors act in the region of the TTS: After knockdown of HIF1 $\alpha$ -AS1, SETDB1 or  
280 MPP8, the chromatin accessibility of both the EPHA2 and ADM transcriptional start sites were  
281 reduced. An increase in accessibility to the region downstream of the EPHA2 TTS was detected (**Fig.**  
282 **6d**). These data indicate that the triplex formation by HIF1 $\alpha$ -AS1 is important for fine-tuning  
283 chromatin accessibility locally and thereby gene expression of EPHA2 and ADM through SETDB1 and  
284 MPP8.

285 **Discussion**

286 The present study combined molecular biology, bioinformatics, physiology and structural analysis to  
287 identify and establish the lncRNA HIF1 $\alpha$ -AS1 as a triplex-forming lncRNA in human endothelial cells.  
288 Through *trans*-acting triplex formation by a specific region within HIF1 $\alpha$ -AS1, EPHA2 and ADM DNA  
289 target sites are primed for their interaction with the HUSH complex members MPP8 and SETDB1 to  
290 mediate gene repression through control of chromatin accessibility. Physiologically, the anti-  
291 angiogenic lncRNA HIF1 $\alpha$ -AS1 is dysregulated in hypoxia and severe angiogenic and pulmonary  
292 diseases like CTEPH, IPAH and GBM. Thus, the present work establishes a putative link of a disease-  
293 relevant lncRNA and the HUSH complex by triplex formation resulting in the inhibition of endothelial  
294 gene expression.

295 The interaction of chromatin modifying complexes with lncRNAs suggests that lncRNAs have  
296 targeting or scaffolding functions within these complexes with the purpose of modulating chromatin  
297 structure and thereby regulating gene expression. Most of these lncRNAs have been identified to  
298 interact with complexes such as PRC2, SWI/SNF, E2F1 and p300, e.g. MEG3<sup>4</sup>, FENDRR<sup>12</sup>, MANTIS<sup>34</sup>  
299 and KHPS1<sup>7,10</sup>. In the present work, we identified other silencing complexes that can be targeted by  
300 lncRNAs: We demonstrated that HIF1 $\alpha$ -AS1 interacts with proteins of the HUSH complex, which  
301 mediates gene silencing. HUSH is also involved in silencing extrachromosomal retroviral DNA<sup>35</sup>.  
302 Recently it has been shown that the HUSH complex, particularly MPP8, which is downregulated in  
303 many cancer types and whose depletion caused overexpression of long interspersed element-1  
304 (LINE-1s) and Long Terminal Repeats, controls type I Interferon signaling involving a mechanism with  
305 dsRNA sensing by MDA5 and RIG-I.<sup>36</sup> Here we report a direct interaction of the HUSH complex  
306 members MPP8 and NP220 with HIF1 $\alpha$ -AS1. Moreover, we identified Exon1 of HIF1 $\alpha$ -AS1 as being  
307 critical for this function. It remains unclear whether the complex exists in its published form in  
308 endothelial cells. Our data propose that, in endothelial cells, the HUSH complex interacts with  
309 H3K9me3 and DNA and that SETDB1 and MPP8, but not NP220, repress gene expression of HIF1 $\alpha$ -  
310 AS1-specific target genes.

311 We propose that HIF1 $\alpha$ -AS1 mediates the anti-angiogenic effects through triplex-formation with the  
312 receptor tyrosine kinase EPHA2 and the preprohormone ADM genes. EPHA2 is a major regulator of  
313 angiogenic processes since EphA2-deficient mice displayed impaired angiogenesis in response to  
314 ephrin-A1 stimulation *in vivo*<sup>37</sup>. EphA2-deficient endothelial cells failed to undergo cell migration and  
315 vascular assembly in response to ephrin-A1 and only adenovirus-mediated transduction of EPHA2  
316 restored the defect<sup>37</sup>. Additionally, the preprohormone ADM promotes arterio- and angiogenesis<sup>29</sup>.  
317 Both genes were upregulated after HIF1 $\alpha$ -AS1 knockdown, explaining why HIF1 $\alpha$ -AS1 knockdown

318 increased sprouting. However, other HIF1 $\alpha$ -AS1 targets are likely to contribute to the phenotype,  
319 such as the proangiogenic genes HIF1 $\alpha$ <sup>38</sup>, THBS1<sup>39</sup>, EGR1<sup>40</sup> or NR2F2<sup>41</sup>.

320 In our unbiased approach, a large number of DNA binding sites were identified for HIF1 $\alpha$ -AS1 with  
321 triplex domain finder analysis. The large number is not unusual as many of these binding sites  
322 overlap and are not identical. Also for other lncRNAs, such as GATA6-AS, FENDRR, HOTAIR and  
323 PARTICLE, many DNA binding sites have been predicted within their target genes<sup>9,14</sup>. EPHA2 and  
324 ADM, as well as PLEC, RP11-276H7.2, MIDN and EGR1 contained a large number of DNA binding sites  
325 for HIF1 $\alpha$ -AS1 and were upregulated after HIF1 $\alpha$ -AS1 knockdown. It is therefore tempting to  
326 speculate that similar regulatory mechanisms may play a role in the regulation of these genes. For  
327 the other target genes, no expression regulation could be found, raising the possibility that DNA  
328 binding of HIF1 $\alpha$ -AS1 could also have unknown effects such as on splicing or the regulation of binding  
329 to promoter elements, histones, transcription factors or 3D chromatin structures.

330 The evidence for triplex formation by HIF1 $\alpha$ -AS1 is based on a number of findings: Firstly, target  
331 recognition by HIF1 $\alpha$ -AS1 occurs via triplex formation involving GA-rich sequences of the DNA targets  
332 and GA-rich sequences within HIF1 $\alpha$ -AS1 lncRNA. This has also been observed for other lncRNAs such  
333 as HOTAIR<sup>42</sup> and MEG3<sup>4</sup>, albeit without using RNAs with different TFR lengths, as was the case here  
334 for HIF1 $\alpha$ -AS1 (27 nt, 46 nt, 131 nt). Secondly, the <sup>1</sup>H-1D NMR and CD spectroscopy data for HIF1 $\alpha$ -  
335 AS1 provided similar but more detailed characteristics for triplex formation, compared with other  
336 studies<sup>4,5</sup>. Through the use of heteroduplex samples, measurements at different temperatures, a  
337 reduction of equivalents of RNA and triplex analysis with stabilized DNA hairpin sequences, our study  
338 allowed an improved and extended analysis of triplex formation. Thirdly, in agreement with previous  
339 work<sup>5</sup>, most of the triplex target sites were located in the promoter region or introns of the DNA  
340 target genes. Fourthly, the triplex formation of HIF1 $\alpha$ -AS1 resulted in gene repression, a finding also  
341 observed for other triplex forming RNAs<sup>3</sup>. We could extend this finding by replacing the TFR2 of  
342 HIF1 $\alpha$ -AS1 with other sequences, which abolished the repressive effects.

343 HIF1 $\alpha$ -AS1 was downregulated in the lungs of patients with specific forms of pulmonary arterial  
344 hypertension (PAH). PAH is characterized by several structural changes, remodelling and lesion  
345 development in the pulmonary arteries. A study by Masri *et al.* demonstrated the impairment of  
346 pulmonary artery endothelial cells from IPAH patients to form tube-like structures<sup>43</sup>. CTEPH, a  
347 complex disorder with major vessel remodeling and small vessel arteriopathy, is characterized by  
348 medial hypertrophy, microthrombi formation and plexiform lesions<sup>44</sup>. It has been further shown that  
349 TGF- $\beta$ -induced angiogenesis was increased by circulating CTEPH microparticles co-cultured with  
350 pulmonary endothelial cells, indicating a pro-angiogenic feedback of endothelial injury<sup>45</sup>. Since  
351 HIF1 $\alpha$ -AS1 knockdown led to an increase in sprouting, we assume that the loss of HIF1 $\alpha$ -AS1 is a

352 compensatory mechanism, which could be putatively included in the above mentioned pro-  
353 angiogenic feedback loop. HIF1 $\alpha$ -AS1 was also reduced in endothelial cells isolated from  
354 glioblastoma. Typically this pathology represents a highly angiogenic situation with defective  
355 endothelium and abnormal morphology<sup>46</sup>. Additionally, HIF1 $\alpha$ -AS1 is pro-apoptotic<sup>26</sup> and so the  
356 reduction of HIF1 $\alpha$ -AS1 could explain the observed sprouting phenotype by the inhibition of  
357 apoptosis. Therefore, it is tempting to speculate that HIF1 $\alpha$ -AS1 harbors atheroprotective roles,  
358 which could be exploited to alter angiogenesis in patients. Strategies to design such therapeutics  
359 require data in other species and in different tissues. HIF1 $\alpha$ -AS1 is not endothelial-specific according  
360 to CAGE analysis. A comprehensive analysis on HIF1 $\alpha$ -AS1 conservation, especially of TFR2, is lacking.  
361 Initial attempts with BLAT showed that the first 1000 nt of the pre-processed HIF1 $\alpha$ -AS1 including  
362 TFR2 were conserved in primates and pigs, but not in rodents (data not shown).

363 Additionally, the data indicates that triplex formation could have therapeutic potential. The single  
364 nucleotide polymorphism (SNP) rs5002 (chr11:10326521 (hg19)) was found within the triplex target  
365 site of ADM with phenoscanner, which lists an association with hemoglobin concentration, red blood  
366 cell count and hematocrit<sup>47</sup>. Another link between a triplex forming lncRNA and PAH was reported by  
367 a massive upregulation of MEG3 in paSMCs from IPAH patients. This prevented hyperproliferation  
368 after MEG3 knockdown and a reduced apoptosis phenotype of IPAH-paSMCs involving a mechanism  
369 with miR-328-3p and IGF1R<sup>48</sup>. Although triplex formation was not studied, another study provided  
370 evidence that a ribonucleotide sequence can be used to form a potential triple helix to inhibit gene  
371 expression of the IGF1R gene in rat glioblastoma cells<sup>49</sup>. MEG3 is known to impair cell proliferation  
372 and to promote apoptosis in glioma cells<sup>50</sup>. This argues that the binding of a lncRNA to DNA is  
373 potentially involved in PAH and GBM.

374 Taken together, the findings presented here highlight a novel pathway of a scaffolding lncRNA within  
375 an epigenetic-silencer complex that has a crucial role in the regulation of endothelial genes.

376 **Online Methods**

377 *Materials*

378 The following chemicals and concentrations were used for stimulation: Human recombinant VEGF-A  
379 165 (R&D, 293-VE), Recombinant Human FGF-basic (154 a.a.) (bFGF, Peprotech, 100-18B), RNase A  
380 (NEB, EN0531) and RNase H (NEB, M0297L). The following antibodies were used: Anti-beta-actin  
381 (Sigma-Aldrich, A1978), Anti-H3-pan (Diagenode, C15200011), Anti-dsDNA [3519 DNA] (Abcam,  
382 ab27156), Anti-DNA-RNA Hybrid [S9.6] (Kerafast, ENH001), Anti-EPHA2 (Bethyl, A302-025-M), Anti-  
383 GAPDH (Sigma, G8795), Anti-HSC70/HSP70 (Enzo Life Sciences, ADI-SPA-820), Anti-MPP8 (Bethyl,  
384 A303-051A-M), Anti-H3K9me3 (Diagenode, SN-146-100), Anti-SETDB1 (Bethyl, A300-121A, for  
385 chromatin immunoprecipitation; Santa Cruz Biotechnology, ESET (G-4): sc-271488, for Proximity  
386 ligation assay) and Anti-ZNF638/NP220 (Bethyl, A301-548A-M).

387 *Cell culture*

388 Pooled human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (CC-2519, Lot  
389 No. 371074, 369146, 314457, 192485, 186864, 171772, Walkersville, MD, USA). HUVECs were  
390 cultured in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. Fibronectin-coated (356009, Corning  
391 Incorporated, USA) dishes were used to culture the cells. Endothelial growth medium (EGM),  
392 consisting of endothelial basal medium (EBM) supplemented with human recombinant epidermal  
393 growth factor (EGF), EndoCGS-Heparin (PeloBiotech, Germany), 8% fetal calf serum (FCS) (S0113,  
394 Biochrom, Germany), penicillin (50 U/mL) and streptomycin (50  $\mu$ g/mL) (15140-122, Gibco/  
395 Lifetechnologies, USA) was used. For each experiment, at least three different batches of HUVEC  
396 from passage 3 were used. In case of hypoxic treatments, cells were incubated in a SciTive  
397 Workstation (Baker Ruskinn, Leeds, UK) at 0.1% O<sub>2</sub> and 5% CO<sub>2</sub> for the times indicated.

398 *Analyses of Triplex-Seq data to identify candidate lncRNAs*

399 Triplex-Seq data of U2OS and HeLa S3 was used from <sup>15</sup>, aligned using STAR<sup>51</sup> and peak-calling  
400 performed with MACS2<sup>52</sup>. Peaks were intersected with Ensembl hg38 gene coordinates to produce a  
401 list of gene-associated peaks, which was filtered for lncRNAs. The overlap of U2OS and HeLa S3  
402 lncRNAs was filtered for high confidence candidates by applying cut-off filters for fold enrichment  
403 (>10) and -log<sub>10</sub>(P) (>20). Next, the candidates were filtered for the presence of a nuclear value (> 0)  
404 in Encode and for the presence of a signal (> 0) in aorta, artery, lymphatic, microvascular, thoracic,  
405 umbilical vein and vein in FANTOM5 CAGE data<sup>19-21</sup>. Subsequently, the remaining candidates (RMRP,  
406 HIF1 $\alpha$ -AS1, RP5-857K21.4, SCARNA2 and SNHG8) were tested for their non-coding probability with  
407 the online tools CPAT<sup>53</sup> and CPC2<sup>54</sup>. Lastly, regions enriched in the Triplex-Seq were manually  
408 inspected in the IGV browser to rule out the possibility that the signals belong to overlapping genes.

409 *Total and nuclear RNA isolation, Reverse transcription and RT-qPCR*

410 Total RNA isolation was performed with the RNA Mini Kit (Bio&Sell). Reverse transcription was  
411 performed with SuperScript III Reverse Transcriptase (Thermo Fisher) and oligo(dT)23 together with  
412 random hexamer primers (Sigma). CopyDNA amplification was measured with RT-qPCR using ITaq  
413 Universal SYBR Green Supermix and ROX as reference dye (Bio-Rad, 1725125) in an AriaMX cyclor  
414 (Agilent). Relative expression of target genes was normalized to  $\beta$ -Actin or 18S ribosomal RNA.  
415 Expression levels were analyzed by the delta-delta Ct method with the AriaMX qPCR software  
416 (Agilent). Oligonucleotides used for amplification are listed in table 1.

417 For nuclear RNA isolation, cells were resuspended in buffer A1 (10 mM HEPES pH 7.6, 10 mM KCl, 0.1  
418 mM EDTA pH 8.0, 0.1 mM EGTA pH 8.0, 1 mM DTT, 40  $\mu$ g/mL PMSF) and incubated on ice for 15 min.  
419 Nonidet was added to a final concentration of 0.75% and cells were centrifuged (1 min, 4  $^{\circ}$ C, 16,000  
420 g). The pellet was washed twice in buffer A1, lysed in buffer C1 (20 mM HEPES pH 7.6, 400 mM NaCl,  
421 1 mM EDTA pH 8.0, 1 mM EGTA pH 8.0, 1 mM DTT, 40  $\mu$ g/mL PMSF) and centrifuged (5 min, 4  $^{\circ}$ C,  
422 16,000 g). The supernatant was used for RNA isolation with RNA Isolation the RNA Mini Kit (Bio&Sell).

423 Table 1. List of primers for qRT-PCR.

Name	Forward Primer (5'-3')	Reverse Primer (5'-3')
b-actin	AAAGACCTGTACGCCAACAC	GTCATACTCCTGCTTGCTGAT
HIF1 $\alpha$ -AS1 (TFR2)	CCGAAATCCCTTCTCAGCAG	TCTGTGTTTAGCGGCGGAGG
HIF1 $\alpha$ -AS1 (E1)	GCCCTCCATGGTGAATCGGTCCCCGCG	CCTTCTCTTCTCCGCGTGTGGAGGGAG
HIF1 $\alpha$ -AS1 (E2)	AGGGCTGTTCCATGTTTAGG	GTCTATGGATGCCACATGC
HIF1 $\alpha$ -AS1 (E1-I)	GCCCTCCATGGTGAATCGGTCCCCGCG	CAACCGAAATCCCTTCTCAGCAGCG
RMRP	TCCGCCAAGAAGCGTATCCC	ACAGCCGCGCTGAGAATGAG
SCARNA2	AGTGTGAGTGGACGCGTGAG	AAGTGTAAGCGGGAGGAGGG
RP5-857K21.4	AGAGTGAGGAGAAGGCTTAC	TTCTGAGTCCCAGAGGTTAC
HIF1 $\alpha$	GCTCATCAGTTGCCACTTCC	ACCAGCATCCAGAAGTTTCC
18S rRNA	CTTTGGTCGCTCGCTCCTC	CTGACCGGTTGGTTTTGAT
HIF1 $\alpha$ -AS1 (TFR1)	TCAGACGAGGCAGCACTGTGCACTGAG G	TCGCTCGCCATTGGATCTCGAGGAACCC
HIF1 $\alpha$ -AS1 (TFR3)	GAGCCCTAATCATAGGACTG	AGGGTCTGAGGTTTGAGTTC
KLF10	AGCCAGCATCCTCAACTATC	GCAGCACTTGCTTTCTCATC
SPHK1	GGAGATGCGCTTCACTCTGG	GGAGGCAGGTGTCTTGGAAC
CSRNP1	TGTGGCTGTCACTGCGATAG	TGTGGTCCATCTGGCACTTG
INTS6	GCCTGGCACCATGTCAGTAG	GCACCAAGGACTCCAGACAC
GATA2	GCAACCCCTACTATGCCAACCC	CAGTGGCGTCTTGAGAAG



IER5	AGACCGGGAACGTGGCTAAC	TCTCAGCACCGGCTTATCGC
YWHAZ	GTGTTCTATTATGAGATTCTGAAC	ATGTCCACAATGTCAAGTTGTCTC
THBS1	TGTACGCCATCAGGGTAAAG	AAGAAGGTGCCACTGAAGTC
EGR1	ACCCAGCAGCCTTCGCTAAC	AGAAGCGGCATCACAGGAC
MIDN	AAGACACCCGGCTCAGTTCCG	TGAGACATGAGGCCCGCTTC
EPHA2	GGCTGAGCGTATCTTCATTG	ACTCGGCATAGTAGAGGTTG
RP11-276H7.2	CCAGACTCCCTTTGCCTACC	GCAGAGAAGACCCACGTACC
PLEC	CCAAGGGCATCTACCAATCC	CACTCCAGCCTCTCAAATCC
ADM	TTCCGTCGCCCTGATGTACC	ATCCGCAGTCCCTCTTCCC
TGFBR1	GAGCGGTCTTGCCCATCTTC	TTCAGGGGCCATGTACCTTTT

424

425 *Knockdown procedures*

426 For small interfering RNA (siRNA) treatments, endothelial cells (80–90% confluent) were transfected  
427 with GeneTrans II according to the instructions provided by MoBiTec (Göttingen, Germany). The  
428 following siRNAs were used: siEPHA2 (Thermo Fisher Scientific, HSS176396), siSETDB1 (Thermo  
429 Fisher Scientific, s19112) and siMPP8 (Thermo Fisher Scientific, HSS123184). As negative control,  
430 scrambled Stealth RNAi™ Med GC (Life technologies) was used. All siRNA experiments were  
431 performed for 48 h.

432 For Locked nucleic acid (LNA)-GapmeR (Exiqon) treatment, the transfection was performed with the  
433 Lipofectamine RNAiMAX (Invitrogen) transfection reagent according to manufacturer's protocol. All  
434 LNA-GapmeR transfections were performed for 48 h. LNA-GapmeRs were designed with the Exiqon  
435 LNA probe designer and contained the following sequences: HIF1 $\alpha$ -AS1 (1) 5'-GAAAGAGCAAGGAAC  
436 A-3' and as a negative Control 5'-AACACGTCTATACGC-3'.

437 *Protein Isolation and Western Analyses*

438 HUVECs were washed in Hanks solution (Applichem) and afterwards lysed with Triton X-100 buffer  
439 (20 mM Tris/HCl pH 7.5, 150 mM NaCl, 10 mM NaPPi, 20 mM NaF, 1% Triton, 2 mM Orthovanadat  
440 (OV), 10 nM Okadaic Acid, protein-inhibitor mix (PIM), 40  $\mu$ g/mL Phenylmethylsulfonylfluorid  
441 (PMSF)). The cells were centrifuged (10 min, 16,000 g) and protein concentration of the supernatant  
442 was determined with the Bradford assay. The cell extract was boiled in Laemmli buffer and equal  
443 amounts of protein were separated with SDS-PAGE. The gels were blotted onto a nitrocellulose  
444 membrane and blocked in Rotiblock (Carl Roth, Germany). After incubation with the first antibody,  
445 infrared-fluorescent-dye-conjugated secondary antibodies (Licor, Bad Homburg, Germany) were used

446 and signals detected with an infrared-based laser scanning detection system (Odyssey Classic, Licor,  
447 Bad Homburg, Germany).

#### 448 *Human Lung samples*

449 The study protocol for tissue donation from human idiopathic pulmonary hypertension patients was  
450 approved by the ethics committee (Ethik Kommission am Fachbereich Humanmedizin der Justus  
451 Liebig Universität Giessen) of the University Hospital Giessen (Giessen, Germany) in accordance with  
452 national law and with Good Clinical Practice/International Conference on Harmonisation guidelines.  
453 Written informed consent was obtained from each individual patient or the patient's next of kin (AZ  
454 31/93, 10/06, 58/15).<sup>55</sup>

455 Human explanted lung tissues from subjects with IPAH, CTEPH or control donors were obtained  
456 during lung transplantation. Samples of donor lung tissue were taken from the lung that was not  
457 transplanted. All lungs were reviewed for pathology and the IPAH lungs were classified as grade III or  
458 IV.

#### 459 *PASMC isolation and culture*

460 Pulmonary arterial smooth muscle cells (PASMCs) were handled and treated as described before<sup>56</sup>.  
461 Briefly, segments of PASMCs, which were derived from human pulmonary arteries (<2 mm in  
462 diameter) of patients with IPAH or from control donors, were cut to expose them to the luminal  
463 surface. Gentle scraping with a scalpel blade was used to remove the endothelium. The media was  
464 peeled away from the underlying adventitial layer. 1-2 mm<sup>2</sup> sections of medial explants were  
465 cultured in Promocell smooth Muscle Cell Growth Medium 2 (Promocell, Heidelberg, Germany). For  
466 each experiment, cells from passage 4-6 were used. A primary culture of human PASMCs was  
467 obtained from Lonza (CC-2581, Basel, Switzerland), grown in SmGM-2 Bulletkit medium (Lonza) and  
468 cultured in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. Cells from passages 4-6 were used for  
469 experiments. For hypoxia experiments, PASMCs were incubated in hypoxia or normoxia chambers for  
470 24 h in hypoxic medium (basal medium containing 1% FCS for human PASMCs). Hypoxia chambers  
471 were equilibrated with a water-saturated gas mixture of 1% O<sub>2</sub>, 5% CO<sub>2</sub>, and 94% N<sub>2</sub> at 37 °C.

#### 472 *Brain microvessel isolation from glioblastoma (GBM) patients*

473 Human Brain microvessel (HMBV) isolation from GBM patients was performed exactly as described  
474 before.<sup>34</sup>

#### 475 *CRISPR/dCas9 activation (CRISPRa) and inactivation (CRISPRi)*

476 Guide RNAs (gRNA) were designed with the help of the web-interfaces of CRISPR design  
477 (<http://crispr.mit.edu/>). CRISPR activation (CRISPRa) was performed with a catalytically inactive Cas9  
478 (dCas9), which is fused to the transcription activator VP64 (pHAGE EF1 $\alpha$  dCas9-VP64), whereas  
479 CRISPRi was performed with a dCas9 fusion to the KRAB repressive domain. Both were used together  
480 with a sgRNA(MS2) vector containing the individual guide RNA (gRNA) to induce or repress HIF1 $\alpha$ -  
481 AS1 gene expression. pHAGE EF1 $\alpha$  dCas9-VP64 and pHAGE EF1 $\alpha$  dCas9-KRAB were a gift from Rene  
482 Maehr and Scot Wolfe (Addgene plasmid # 50918, # 50919)<sup>57</sup> and sgRNA(MS2) cloning backbone was  
483 a gift from Feng Zhang (Addgene plasmid # 61424)<sup>58</sup>. The following oligonucleotides were used for  
484 cloning of the guide RNAs into the sgRNA(MS2) vector: For CRISPRa of HIF1 $\alpha$ -AS1 5'-CACCGGGGC  
485 CGGCCTCGGCGTTAAT-3' and 5'-AAACATTAACGCCGAGGCCGCC-3', and for CRISPRi of HIF1 $\alpha$ -AS1  
486 5'-CACCGGTCTGGTGAGGATCGCATGA-3' and 5'-AAACTCATGCGATCCTCACCAGACC-3'. After cloning,  
487 plasmids were purified and sequenced. The transfection of the plasmids in HUVEC was performed  
488 using the NEON electroporation system (Invitrogen).

#### 489 *CRISPR-Cas9 genome editing*

490 For genome editing, the ArciTect Cas9-eGFP system was used according to the manufacturer's  
491 conditions (STEMCELL Technologies, Köln, Germany). Briefly, ArciTect™ CRISPR-Cas9 RNP Complex  
492 solution was generated with 60  $\mu$ M gRNA and tracrRNA and 3.6  $\mu$ g ArciTect™ Cas9-eGFP Nuclease.  
493 Afterwards, 20  $\mu$ M single-strand oligodeoxynucleotide (ssODN) was added to the RNP solution. The  
494 following gRNA was used to target TFR2 of HIF1 $\alpha$ -AS1: 5'-ACGTGCTCGTCTGTGTTTAG-3'. The  
495 following ssODNs (Integrated DNA Technologies, Leuven, Belgium) were used to replace TFR2: MEG3,  
496 5'-GAGGCACAGCTGGGACGGGCTGCGACGCTCACGTGCTCGTCTGTGTTGTAATCGCTCCCTCT  
497 CTGCTCTCCGATGGGGGTGCGGCTCAGCCCGAGTCTGGGGACTCTGCGCCTTCTCCGAAGGAA  
498 GGCGG-3', negative control Luc 5'-GCTGAGGCACAGCTGGGACGGGCTGCG  
499 ACGTACAGTCTGCTCGTCTGTGTTGTAATTATCACGCTCGTCTCGTTCGGTATGATGGGGGTGCGGCT  
500 CAGCCCGAGTCTGGGGACTCTGCGCCTTCTCCGAAGGAAG-3'. 400.000 HUVECs were seeded in  
501 a 12-well plate and electroporated in E2 buffer with the NEON electroporation system (Invitrogen)  
502 (1,400 V, 1x 30 ms pulse). A full medium exchange was done every 24 h and cells were incubated for  
503 72 h.

#### 504 *HIF1 $\alpha$ -AS1 mutants and pCMV6-MPP8-10xHis*

505 To clone pcDNA3.1+HIF1 $\alpha$ -AS1, HIF1 $\alpha$ -AS1 was amplified with PCR from cDNA (forward primer: 5'-  
506 ATATTAGGTACCCGCCGCCGCGCCCTCCATGGTG-3', reverse primer: 5'-ACGGGAATTCTAATGGAACAT  
507 TTCTTCTCCCTAG-3') and insert and vector (pcDNA3.1+) were digested with Acc65I/EcoRI and ligated.  
508 pCMV6-MPP8-MYC-DDK was obtained from Origene (#RC202562L3).

509 To create pcDNA3.1+HIF1-AS1- $\Delta$ exon1 (1-116), pcDNA3.1+HIF1-AS1- $\Delta$ exon2 (117-652),  
510 pcDNA3.1+HIF1-AS1- $\Delta$ exon1 (26-78) and pCMV6-MPP8-10xHIS (replacement of c-terminally MYC-  
511 DDK by 10xHIS), site-directed mutagenesis was performed with the Q5 Site-Directed Mutagenesis Kit  
512 (NEB) according to the instructions of the manufacturer. Oligonucleotides and annealing  
513 temperatures for mutagenesis were calculated with the NEBaseChanger online tool from NEB. The  
514 pcDNA3.1+HIF1 $\alpha$ -AS1 and pCMV6-MPP8-Myc-DDK plasmids served as templates and were amplified  
515 with PCR with the following oligonucleotides to obtain the individual constructs: for  
516 pcDNA3.1+HIF1 $\alpha$ -AS1- $\Delta$ exon1 (1-116), 5'-ACTACAGTTCAACTGTCAATTG-3' and 5'-  
517 GGTACCAAGCTTAAGTTTAAAC-3', for pcDNA3.1+HIF1-AS1- $\Delta$ exon2 (117-652), 5'-  
518 GAATTCTGCAGATATCCAG-3' and 5'-CTTTCCTTCTCTTCTCCG-3', for pcDNA3.1+HIF1 $\alpha$ -AS1- $\Delta$ exon1 (26-  
519 78), 5'-AGCGCTGGCTCCCTCCAC-3' and 5'-TTCACCATGGAGGGCGCC-3', for pCMV6-MPP8-10xHIS, 5'-  
520 CACCATCATCACCACCATCACTAAACGGCCGCGCCGCGGTCAT-3' and 5'-  
521 GTGATGGTGAGAGCCTCCACCCCTGCAGCTGCACTCTGTATGCACCTATTAGC-3'. The plasmids were  
522 verified by sequencing.

523 To generate purified MPP8-10xHIS protein, pCMV6-MPP8-10xHIS was overexpressed in HEK293 with  
524 Lipofectamine 2000 according to the manufacturer's protocol. Cells were lysed with three cycles  
525 snap freezing in nitrogen and 2% triton X-100 with protease inhibitors. Recombinant MPP8-10xHis  
526 was purified using HisTrap FF crude columns (Cytiva Europe, Freiburg, Germany, #11000458) with a  
527 linear gradient of imidazole (from 20 to 500 mM, Merck, Burlington, United States, #104716) in an  
528 Äkta Prime Plus FPLC system (GE Healthcare/Cytiva Europe).

#### 529 *In vitro transcription and RNA 3'end biotinylation*

530 Prior to *in vitro* transcription, pcDNA3.1+HIF1 $\alpha$ -AS1, pcDNA3.1+HIF1 $\alpha$ -AS1- $\Delta$ exon1 (1-116),  
531 pcDNA3.1+HIF1 $\alpha$ -AS1- $\Delta$ exon2 (117-652), pcDNA3.1+HIF1 $\alpha$ -AS1- $\Delta$ exon1 (26-78) or control pcDNA3.1+  
532 were linearized with SmaI (Thermo Fisher, FD0663). After precipitation and purification of linearized  
533 DNA, DNA was *in vitro* transcribed according to the manufacturers protocol with T7 Phage RNA  
534 Polymerase (NEB), and DNA was digested with RQ DNase I (Promega). The remaining RNA was  
535 purified with the RNeasy Mini Kit (Qiagen) and used for binding reactions with MPP8-10xHis in RIP  
536 experiments. For RNA pulldown experiments, RNA of HIF1 $\alpha$ -AS1 or of the control pcDNA3.1+ were  
537 further biotinylated at the 3'end with the Pierce RNA 3'end biotinylation kit (Thermo Fisher).

#### 538 *RNA pulldown assay and mass spectrometry*

539 The RNA pulldown assay was performed similar to<sup>34</sup>. For proper RNA secondary structure formation,  
540 150 ng of 3'end biotinylated HIF1 $\alpha$ -AS1 or control RNA was heated for 2 min at 90 °C in RNA folding  
541 buffer (10 mM Tris pH 7.0, 0.1 M KCl, 10 mM MgCl<sub>2</sub>), and then put on RT for 20 min. 1x10<sup>7</sup> HUVECs

542 were used per sample. Isolation of nuclei was performed with the truCHIP™ Chromatin Shearing Kit  
543 (Covaris, USA) according to the manufacturers protocol without shearing the samples. Folded Bait  
544 RNA was incubated in nuclear cell extracts for 3 h at 4 °C. After incubation, samples were UV  
545 crosslinked. Afterwards, Streptavidin M-270 Dynabeads (80  $\mu$ L Slurry, Thermo Fisher) were incubated  
546 with cell complexes for 2 h at 4 °C. After 4 washing steps with the lysis buffer of the truCHIP  
547 chromatin Shearing Kit (Covaris, USA), beads were put into a new Eppendorf tube. For RNA analysis,  
548 RNA was extracted with TRIzol (Thermo Fisher). Afterwards, RNA purification was performed with  
549 the RNeasy Mini Kit (Qiagen). If indicated, RT-qPCR was performed. For mass spectrometric  
550 measurements in order to reduce complexity, samples were eluted stepwise from the beads.

551 Method description and mass spectrometry proteomics data have been deposited to the  
552 ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner  
553 repository<sup>59</sup> with the dataset identifier PXD023512. Therefore the samples were labelled H1-H5 for  
554 HIF1 $\alpha$ -AS1 and C1-C5 for the negative control RNA.

#### 555 *RNA immunoprecipitation*

556  $1 \times 10^7$  HUVECs were used per sample. Nuclei isolation was performed with the truCHIP™ Chromatin  
557 Shearing Kit (Covaris, USA) according to the manufacturers protocol without shearing the samples.  
558 After pre-clearing with 20  $\mu$ L DiaMag Protein A and Protein G (Diagenode), 10% of the pre-cleared  
559 sample served as input and the lysed nuclei were incubated with the indicated antibody or IgG alone  
560 for 12 h at 4 °C. The complexes were then incubated with 50  $\mu$ L DiaMag Protein A and Protein G  
561 (Diagenode) beads for 3 h at 4 °C, followed by 4 washing steps in Lysis Buffer from the truCHIP™  
562 Chromatin Shearing Kit (Covaris, USA). In case of RNase treatments, the samples were washed once  
563 in TE-buffer and then incubated for 30 min at 37 °C in buffer consisting of 50 mM Tris-HCl pH 7.5-8.0,  
564 150 mM NaCl, 1 mM MgCl<sub>2</sub> containing 2  $\mu$ L RNase H per 100  $\mu$ L buffer. Afterwards the samples were  
565 washed in dilution buffer (20 mmol/L Tris/HCl pH 7.4, 100 mmol/L NaCl, 2 mmol/L EDTA, 0.5% Triton  
566 X-100, 1  $\mu$ L Superase In (per 100  $\mu$ L) and protease inhibitors). Prior to elution, beads were put into a  
567 new Eppendorf tube. RNA was extracted with TRIzol (Thermo Fisher) followed by RNA purification  
568 with the RNeasy Mini Kit (Qiagen), reverse transcription and qRT-PCR.

569 For the *in vitro* RIP assay, the individual RNAs were folded as mentioned above in RNA folding buffer  
570 (10 mM Tris pH 7.0, 0.1 M KCl, 10 mM MgCl<sub>2</sub>), and then put on RT for 20 min. The binding reaction  
571 with purified MPP8-10xHIS was performed for 2 h at 4 °C in binding buffer (20 mmol/L Tris/HCl  
572 pH8.0, 150 mmol/L KCl, 2 mmol/L EDTA pH 8.0, 5 mmol/L MgCl<sub>2</sub>, 2  $\mu$ L/mL Superase In and protease  
573 inhibitors). After pre-clearing with 20  $\mu$ L DiaMag Protein A and Protein G (Diagenode), 5% of the pre-  
574 cleared sample served as input. The mixture was incubated with an MPP8 antibody for 3 h at 4 °C.

575 The complexes were then incubated with 50  $\mu$ L DiaMag Protein A and Protein G (Diagenode) beads  
576 for 1 h at 4  $^{\circ}$ C, followed by 4 washing steps (5 min, 4  $^{\circ}$ C, each) in binding buffer. Elution, RNA  
577 extraction and RT-qPCR were performed as mentioned above. RT-qPCR was performed with primers  
578 targeting the MCS within the in vitro transcribed sequences before (5'-GTGCTGGATATC  
579 TGCAGAATTC-3') and after (5'-GTGCTGGATATCTGCAGAATTC-3') the HIF1 $\alpha$ -AS1 sequences.

#### 580 *Assay for Transposase Accessibility (ATAC)-Sequencing*

581 ATAC-Seq was performed similar to<sup>34</sup>. 100.000 HUVECs were used for ATAC library preparation using  
582 Tn5 Transposase from Nextera DNA Sample Preparation Kit (Illumina). Cell pellets were resuspended  
583 in 50  $\mu$ L PBS and mixed with 25  $\mu$ L TD-Buffer, 2.5  $\mu$ L Tn5, 0.5  $\mu$ L 10% NP-40 and 22  $\mu$ L H<sub>2</sub>O. The  
584 mixture was incubated at 37  $^{\circ}$ C for 30 min followed by 30 min at 50  $^{\circ}$ C together with 500 mM EDTA  
585 pH 8.0 for optimal recovery of digested DNA fragments. 100  $\mu$ L of 50 mM MgCl<sub>2</sub> was added for  
586 neutralization. The DNA fragments were purified with the MinElute PCR Purification Kit (Qiagen).  
587 Amplification of library together with indexing was performed as described elsewhere<sup>60</sup>. Libraries  
588 were mixed in equimolar ratios and sequenced on NextSeq500 platform using V2 chemistry and  
589 assessed for quality by FastQC. Reaper version 13-100 was employed to trim reads after a quality  
590 drop below a mean of Q20 in a window of 5 nt<sup>61</sup>. Only reads above 15 nt were cleared for further  
591 analyses. These were mapped versus the hg19 version of the human genome with STAR 2.5.2b using  
592 only unique alignments to exclude reads with uncertain arrangement. Reads were further  
593 deduplicated using Picard 2.6.0 (Picard: A set of tools (in Java)<sup>62</sup> for working with next generation  
594 sequencing data in the BAM format) to avoid PCR artefacts leading to multiple copies of the same  
595 original fragment. The Macs2 peak caller (version 2.1.0)<sup>52</sup> as employed in punctate mode to  
596 accommodate for the range of peak widths typically expected for ATAC-seq. The minimum qvalue  
597 was set to -4 and FDR was changed to 0.0001. Peaks overlapping ENCODE blacklisted regions (known  
598 misassemblies, satellite repeats) were excluded. Peaks were annotated with the promoter (TSS +/-  
599 5000 nt) of the gene most closely located to the centre of the peak based on reference data from  
600 GENCODE v19. To compare peaks in different samples, significant peaks were overlapped and unified  
601 to represent identical regions. The counts per unified peak per sample were computed with  
602 BigWigAverageOverBed (UCSC Genome Browser Utilities,  
603 <http://hgdownload.cse.ucsc.edu/downloads.html>). Raw counts for unified peaks were submitted to  
604 DESeq2 (version 1.14.1) for normalization<sup>63</sup>. Spearman correlations were produced to identify the  
605 degree of reproducibility between samples using R. To permit a normalized display of samples in IGV,  
606 the raw BAM files were normalized for sequencing depth (number of mapped deduplicated reads per  
607 sample) and noise level (number of reads inside peaks versus number of reads not inside peaks). Two

608 factors were computed and applied to the original BAM files using bedtools genomecov resulting in  
609 normalized BigWig files.

610 For samples used after siRNA-mediated silencing of MPP8 and SETDB1 as well as the corresponding  
611 LNA GapmeR knockdown of HIF1 $\alpha$ -AS1, the improved OMNI-ATAC protocol<sup>64</sup> was used and samples  
612 were sequenced on a Nextseq2000. The resulting data were trimmed and mapped using Bowtie2<sup>65</sup>.  
613 Data were further processed using deepTools<sup>66</sup>. For visualization, the Integrative Genomics Viewer<sup>67</sup>  
614 was used.

#### 615 *Electrophoretic mobility shift assay (EMSA)*

616 RNA transcripts corresponding to HIF1 $\alpha$ -AS1 TFR2 region were produced by *in vitro* transcription  
617 using the MEGAscript T7 Transcription Kit (Invitrogen) with DNA templates containing the T7  
618 promoter and the sequence to be transcribed. The 131 nt template was produced by PCR using  
619 genomic DNA and sequence specific primers, of which the forward one contains the T7 promoter as  
620 extension. The DNA templates for the 27 nt and 46 nt transcripts were created by hybridization of  
621 single stranded oligos (Sigma) creating a partially (at the T7 promoter sequence) double-stranded  
622 molecule.

623 Triplex target DNA was created by hybridization of equimolar concentrations of short  
624 complementary DNA oligos corresponding to the target region in question, whereby only the purine-  
625 rich one was <sup>32</sup>P- $\gamma$ ATP-end labelled using T4 PNK enzyme and cleaned with Ethanol precipitation to  
626 remove unincorporated hot ATP. This strategy avoids visualization of any RNA:DNA hybrids, that may  
627 occur between single stranded molecules. The two oligos were then heated to 70 °C for 10 min after  
628 which gradually decreasing the temperature (0.1 °C/sec) to 20 °C, in a buffer containing 10 mM Tris-  
629 acetate pH 7.4, 5 mM MgOAc and 50 mM NaCl.

630 For triplex formation, different amounts of the respective RNA transcripts (50-250 pmol, as  
631 indicated) were incubated in a 10  $\mu$ L reaction with 0.25 pmol of radiolabeled duplex oligos for 1 h at  
632 37 °C in 40 mM Tris-acetate pH 7.4, 30 mM NaCl, 20 mM KCl, 5 mM MgOAc, 10% glycerol and  
633 PhosSTOP EASYpack (Roche). For monitoring of triplex formation, the reactions were loaded on a  
634 12% polyacrylamide-bisacrylamide gel containing 40 mM Tris-Ac pH 7.4 and 5 mM MgOAc and run at  
635 120V for 2-3 h at RT. The gels were subsequently dried and exposed a phosphoimager screen  
636 overnight, which was then scanned in Fujifilm BAS 1800-II Phosphoimager using the BAS reader 2.2.6  
637 software. Triplex formation was observed as an RNA-dependent shift of the hot duplex oligo as a  
638 result of its binding by the RNA and thus slower migration.

639 Specific sequences for EMSA design and oligonucleotide preparation are shown in tables 2-4.

640 Table 2. DNA oligos used for triplex target sites.

Name	Sequence (5'-3')
EPHA2_3_GA	AGAGGGTAAGGAGATAGGAGAAACC
EPHA2_3_CT	GGTTTCTCCTATCTCCTTACCCTCT

641

642 Table 3. Oligos for generation of the DNA template by PCR for *in vitro* transcription of RNA (131mer).

Name	Sequence (5'-3')
T7 F primer	TAATACGACTCACTATAGGGTGTTTAGCGGCGGAGGAAAG
HIF1 $\alpha$ -AS1 R primer	AACCGAAATCCCTTCTCAGCA
PCR product	TAATACGACTCACTATAGGGTGTTTAGCGGCGGAGGAAAGAGAAAGGAGATGGG GGTGC GGCTCAGCCGAGTCTGGGACTCTGCGCCTTCTCCGAAGGAAGGCGGTG CCCGGCTTTGGGAGGCGCTGCTGAGAAGGGATTTGGTT
Resulting sequence (131mer)	GGGTGTTTAGCGGCGGAGGAAAGAGAAAGGAGATGGGGGTGCGGCTCAGCCCGA GTCTGGGACTCTGCGCCTTCTCCGAAGGAAGGCGGTGCCCGGCTTTGGGAGGCG CTGCTGAGAAGGGATTTGGTT

643

644 Table 4. Oligos for generation of partially double stranded DNA template for *in vitro* transcription of  
645 RNA (27mer, 46mer).

Name	Sequence (5'-3')
T7 oligo short	TAATACGACTCACTATAGGG
Template-27nt	CTCCTTTCTTTCTCCGCCGCTCTCCCTATAGTGAGTCGTATTA
Resulting sequence (27mer)	GGGGAGGAAAGAGAAAGGAGATGGGGG
T7 oligo long	TAATACGACTCACTATAGGGAGA
Template-46nt	CGCACCCCATCTCCTTTCTTTCTCCGCCGCTAAACATCTCCCTATAGTGAGTCGTA TTA
Resulting sequence (46mer)	GGGAGATGTTTAGCGGCGGAGGAAAGAGAAAGGAGATGGGGGTGCG

646

#### 647 RNA and DNA Hybridization

648 By hybridization of the RNA strand to the DNA duplex or DNA hairpin DNA:DNA:RNA triplexes were  
649 formed. First the complementary DNA single strands were incubated at 95 °C for 5 min in  
650 hybridization buffer (25 mM HEPES, 50 mM NaCl, 10 mM MgCl<sub>2</sub> (pH 7.4)) and afterwards cooled  
651 down to RT. Triplex formation was performed by adding RNA to previously hybridized double  
652 stranded DNA for 1 h at 60 °C and then cooled down to RT.<sup>13</sup> For the <sup>1</sup>H-1D NMR, CD and melting  
653 curve experiments, the HIF1 $\alpha$ -AS1-TFR2 (TFO2-23) sequence 5'-GCG GCGGAGGAAAGAGAAAGGAG-3'  
654 (length 23nt, GC=50.9%) was used in combination with the DNA sequences listed in table 5.



655 Table 5. DNA oligos used for <sup>1</sup>H-1D NMR, CD and melting curve analysis analysis.

Name	Sequence (5'-3')	size	Genomic location (hg19)
EPHA2 (GA-rich)	GGTTTCTCCTATCTCCTTACCCTCT	25nt	chr1:16,478,543-16,478,567
EPHA2 (CT-rich)	AGAGGGTAAGGAGATAGGAGAAACC	25nt	chr1:16,478,543-16,478,567
EPHA2-hairpin	GGTTTCTCCTATCTCCTTACCCTCTTTTTT AGAGGGTAAGGAGATAGGAGAAACC	55nt	chr1:16,478,543-16,478,567
ADM (CT-rich)	TCTTTCCTCAGCCAC	15nt	chr11:10,326,521-10,326,535
ADM (GA-rich)	GTGGCTGAGGAAAGA	15nt	chr11:10,326,521-10,326,535
ADM-hairpin	TCTTTCCTCAGCCACTTTTTGTGGCTGAG GAAAGA	35nt	chr11:10,326,521-10,326,535

656

### 657 *CD spectroscopy and melting curve analysis*

658 Circular dichroism spectra were acquired on a Jasco J-810 spectropolarimeter. The measurements  
659 were recorded from 210 to 320 nm at 25 °C using 1 cm path length quartz cuvette. CD spectra were  
660 recorded on 8  $\mu$ M samples of each DNA duplex, DNA:RNA heteroduplex and DNA:DNA:RNA-triplex in  
661 25 mM HEPES, 50 mM NaCl, 10 mM MgCl<sub>2</sub> (pH 7.4). Spectra were acquired with 8 scans and the data  
662 was smoothed with Savitzky-Golay filters. Observed ellipticities recorded in millidegree (mdeg) were  
663 converted to molar ellipticity  $[\theta] = \text{deg} \times \text{cm}^2 \times \text{dmol}^{-1}$ . Melting curves were acquired at constant  
664 wavelength using a temperature rate of 1 °C/min in a range from 5 °C to 95 °C. All data were  
665 evaluated using SigmaPlot 12.5.

### 666 *NMR spectroscopy*

667 All NMR samples were prepared in NMR buffer containing 25 mM HEPES-d18, 50 mM NaCl, 10 mM  
668 MgCl<sub>2</sub> (pH 7.4) with addition of 5 to 10% D2O. All samples were internally referenced with 2,2-  
669 dimethyl-2-silapentane-5-sulfonate (DSS). The final NMR sample concentrations ranged between 50  
670  $\mu$ M to 300  $\mu$ M. NMR spectra were recorded in a temperature range from 278 K to 308 K on Bruker  
671 600, 800, 900 and 950 MHz spectrometers. <sup>1</sup>H NMR spectra were recorded with jump-return-Echo<sup>68</sup>  
672 and gradient-assisted excitation sculpting<sup>69</sup> for water suppression. NMR data was collected,  
673 processed and analyzed using TopSpin 3.6.2 (Bruker).

### 674 *Spheroid outgrowth assay*

675 Spheroid outgrowth assays in HUVEC were performed as described in<sup>70</sup>. Stimulation of Spheroids was  
676 performed with the indicated amounts of VEGF-A 165 or bFGF for 16 h. Images were generated with  
677 an Axiovert135 microscope (Zeiss). Sprout numbers and cumulative sprout lengths were quantified  
678 by analysis with the AxioVision software (Zeiss).

679 *Proximity ligation assay (PLA)*

680 The PLA was performed as described in the manufacturer's protocol (Duolink II Fluorescence, OLink,  
681 Upsalla, Sweden). HUVECs were fixed in phosphate buffered formaldehyde solution (4%),  
682 permeabilized with Triton X-100 (0.2%), blocked with serum albumin solution (3%) in phosphate-  
683 buffered saline, and incubated overnight with anti-MPP8, anti-dsDNA, anti-SETDB1 or anti-H3K9me3  
684 antibodies. Samples were washed and incubated with the respective PLA-probes for 1 h at 37 °C.  
685 After washing, samples were ligated for 30 min (37 °C). After an additional washing step, the  
686 amplification with polymerase was performed for 100 min (37 °C). The nuclei were stained using  
687 DAPI. Images (with Alexa Fluor, 546 nm) were acquired by confocal microscope (LSM 510, Zeiss).

688 *Chromatin Immunoprecipitation*

689 Preparation of HUVEC extracts, crosslinking and isolation of nuclei was performed with the truCHIP™  
690 Chromatin Shearing Kit (Covaris, USA) according to the manufacturers protocol. The procedure was  
691 similar to <sup>71</sup>. The lysates were sonified with the Bioruptur Plus (10 cycles, 30 s on, 90 s off, 4 °C;  
692 Diagenode, Seraing, Belgium). Cell debris was removed by centrifugation and the lysates were diluted  
693 1:3 in dilution buffer (20 mmol/L Tris/HCl pH 7.4, 100 mmol/L NaCl, 2 mmol/L EDTA, 0.5% Triton X-  
694 100 and protease inhibitors). Pre-clearing was done with DiaMag protein A and protein G coated  
695 magnetic beads (Diagenode, Seraing, Belgium) for 1 h at 4 °C. The samples were incubated over night  
696 at 4 °C with the antibodies indicated. 5% of the samples served as input. The complexes were  
697 collected with 50  $\mu$ L DiaMag protein A and protein G coated magnetic beads (Diagenode, Seraing,  
698 Belgium) for 3 h at 4 °C, washed twice for 5 min with each of the wash buffers 1-3 (Wash Buffer 1: 20  
699 mmol/L Tris/HCl pH 7.4, 150 mmol/L NaCl, 0.1% SDS, 2 mmol/L EDTA, 1% Triton X-100; Wash Buffer  
700 2: 20 mmol/L Tris/HCl pH 7.4, 500 mmol/L NaCl, 2 mmol/L EDTA, 1% Triton X-100; Wash Buffer 3: 10  
701 mmol/L Tris/HCl pH 7.4, 250 mmol/L lithium chloride, 1% Nonidet p-40, 1% sodium deoxycholate, 1  
702 mmol/L EDTA) and finally washed with TE-buffer pH 8.0. In case of RNase treatments, the samples  
703 were washed once in TE-buffer and then incubated for 30 min at 37 °C in buffer consisting of 50 mM  
704 Tris-HCl pH 7.5-8.0, 150 mM NaCl, 1 mM MgCl<sub>2</sub> containing 2  $\mu$ L RNase H or 2  $\mu$ L RNase A per 100  $\mu$ L  
705 buffer. Elution of the beads was done with elution buffer (0.1 M NaHCO<sub>3</sub>, 1% SDS) containing 1x  
706 Proteinase K (Diagenode, Seraing, Belgium) and shaking at 600 rpm for 1 h at 55 °C, 1 h at 62 °C and  
707 10 min at 95 °C. After removal of the beads, the eluate was purified with the QiaQuick PCR  
708 purification kit (Qiagen, Hilden, Germany) and subjected to qPCR analysis. As a negative control  
709 during qPCR, primer for the promoter of GAPDH were used. The primers are listed in table 6.

710 Table 6. List of primers for ChIP-qPCR.

Name	Forward Primer (5'-3')	Reverse Primer (5'-3')
GAPDH promoter	TGGTGTCAGGTTATGCTGGGCCAG	GTGGGATGGGAGGGTGTCTGAACAC
EPHA2 TTS	CAGGTAGCTGCCAATAAGTG	AGGGCTTTACCCTCTGAATC
ADM TTS	CGCGTGGCTGAGGAAAGAAAGG	GCTTTATAAGCGCACGGGTGGG

711

### 712 *Triplex domain finder analysis*

713 Triplex formation of *HIF1 $\alpha$ -AS1* was predicted using the Triplex Domain Finder (TDF)<sup>14</sup> with the  
714 human pre-spliced *HIF1 $\alpha$ -AS1* sequence (NR\_047116.1, gene ID 100750246) to target DNA regions  
715 around genes with ATAC-Seq peaks upon HIF1 $\alpha$ -AS1 silencing. For annotation of HIF1 $\alpha$ -AS1 triplex  
716 forming regions across DNA triplex target sites, genome version hg19 was used. Randomization was  
717 performed for 200 times. Enrichment was given at a p-value <0.05.

### 718 *Data availability*

719 ATAC-Seq data was uploaded to the NCBI SRA database (PRJNA765209, while it remains in private  
720 status upon request).

721 For data about HIF1 $\alpha$ -AS1 interaction partners identified with mass spectrometry, the data and  
722 methods were uploaded with the dataset identifier PXD023512 to PRIDE  
723 (<http://www.ebi.ac.uk/pride>) and remain in private status upon request.

### 724 *Publicly available datasets used*

725 Triplex-Seq data was used from<sup>15</sup>. Fantom5 Encode CAGE expression data was obtained from  
726 FANTOM5 website (Gencode v19).<sup>19-21</sup> ChIP-Seq datasets for HUVEC H3K4me3, H3K27Ac and H3K9Ac  
727 were taken from Encode<sup>72</sup>.

### 728 *Statistics*

729 Unless otherwise indicated, data are given as means  $\pm$  standard error of mean (SEM). Calculations  
730 were performed with Prism 8.0 or BiAS.10.12. The latter was also used to test for normal distribution  
731 and similarity of variance. In case of multiple testing, Bonferroni correction was applied. For multiple  
732 group comparisons ANOVA followed by post hoc testing was performed. Individual statistics of  
733 dependent samples were performed by paired t-test, of unpaired samples by unpaired t-test and if  
734 not normally distributed by Mann-Whitney test. P values of <0.05 was considered as significant.  
735 Unless otherwise indicated, n indicates the number of individual experiments.

736 **Acknowledgments**

737 We thank Cindy F. Höper for excellent technical assistance and Jana Meisterknecht for help with  
738 mass spectrometry. We are grateful to Katalin Pálfi and Tanja Lüneburg for the help with cell culture.

739 This work was supported by the Goethe University Frankfurt am Main, the German Centre for  
740 Cardiovascular Research (DZHK, Funding Postdoc start up, Förderkennzeichen: 81X3200107), the DFG  
741 excellence cluster Cardiopulmonary Institute (CPI) EXS2026 and the DFG Transregio TRR267 (TP A04,  
742 TP A06 and TP Z02). Work at BMRZ is supported by the state of Hesse.

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751 HS and RPB wrote the manuscript. All authors interpreted the data and approved the manuscript.

752 **Competing interests**

753 The authors have declared that no conflict of interest exists.

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940 **Figure legends**

941 **Fig. 1: HIF1 $\alpha$ -AS1 is a triplex- and DNA-associated RNase H-insensitive lncRNA in endothelial cells.**

942 **a**, Overview of the identification of endothelial-expressed triplex-forming lncRNAs. lncRNAs from a  
943 previous Triplex-Seq study in HeLa S3 and U2OS were overlapped, filtered with high stringency and  
944 analyzed for nuclear expression in endothelial cells with Encode and FANTOM5 CAGE data followed  
945 by analyses for noncoding probability and enriched peaks in the Triplex-Seq data. **b**, RNA-  
946 immunoprecipitation with anti-dsDNA followed by qPCR (RIP-qPCR) targeting the lncRNA candidates  
947 in HUVEC. Samples were treated with or without RNase H.  $\beta$ Act served as control for RNase H-  
948 mediated degradation. n=3. **c**, Scheme of the human genomic locus of HIF1 $\alpha$ -AS1. **d**, RT-qPCR after  
949 anti-dsDNA-RIP in HUVEC. HIF1 $\alpha$  and 18S rRNA served as negative control. One-way ANOVA with  
950 Tukey's post hoc test, n=3. **e**, RIP-qPCR with anti-histone3 (H3) in HUVEC. Data was normalized  
951 against GAPDH. Paired t-test, n=4. **f**, RT-qPCR of HIF1 $\alpha$ -AS1 in HUVEC treated with hypoxia (0.1% O<sub>2</sub>)  
952 for the indicated time points. Normoxia served as negative control (CTL). n=3, One-Way ANOVA with  
953 Bonferroni post hoc test. **g**, RT-qPCR of HIF1 $\alpha$ -AS1 in HUVECs treated with hypoxia (0.1% O<sub>2</sub>) followed  
954 by reoxygenation with normoxia (after 24 h hypoxia) for the indicated time points. n=6, One-Way  
955 ANOVA with Dunnett's post hoc test. **h**, RT-qPCR of HIF1 $\alpha$ -AS1 in lungs from control donors (CTL,  
956 n=6) or patients with idiopathic pulmonary arterial hypertension (IPAH, n=6) or chronic  
957 thromboembolic pulmonary hypertension (CTEPH, n=8). One-Way ANOVA with Tukey's post hoc test.  
958 Error bars are defined as mean +/- SEM. \*p<0.05.

959 **Fig. 2: HIF1 $\alpha$ -AS1 potentially forms DNA:DNA:RNA triplexes.**

960 **a**, Overview of the identification of HIF1 $\alpha$ -AS1 triplex forming regions (TFR) and their DNA triplex target sites (TTS) with triplex domain  
961 finder (TDF). HIF1 $\alpha$ -AS1 pre-RNA and ATAC-Seq of HUVECs treated with or without LNA GapmeRs  
962 against HIF1 $\alpha$ -AS1 were used as input. RIP and LNA GapmeRs were used to validate the findings  
963 obtained by TDF. **b**, Number of triplex target regions of three statistically significant TFRs of HIF1 $\alpha$ -  
964 AS1 identified with TDF. Numbers in brackets represent the position of the individual TFR within  
965 HIF1 $\alpha$ -AS1 pre-RNA. All TFRs have a significantly higher number of triplex target regions in targets  
966 (blue) than non-target regions (grey). **c**, Circos plot showing the localization of the individual TFR  
967 within HIF1 $\alpha$ -AS1 pre-RNA and its interaction with the chromosomal TTS. **d**, Overlap of TTS of the  
968 individual TFRs of HIF1 $\alpha$ -AS1. **e**, Identification of RNase H-resistant TFRs. RIP with S9.6 RNA/DNA  
969 hybrid antibody with or without RNase H treatment in HUVEC followed by qPCR for the TFRs. Ratio of  
970 %-input recovery with/without RNase H treatment is shown. n=8, paired t-test. **f**, HIF1 $\alpha$ -AS1 TFR2 top  
971 target genes, their genomic location and number of TTS identified by TDF. **g**, RT-qPCR of triplex target  
972 genes of TFR2 after knockdown of HIF1 $\alpha$ -AS1 in HUVEC. n=6, One-Way ANOVA with Holm's Sidak  
973 post hoc test. **h**, Three different triplex target regions of HIF1 $\alpha$ -AS1 are shown. Triplex target regions  
974 are highlighted in grey, triplex target sites are shown in blue. Error bars are defined as mean +/- SEM.  
975 \*p<0.05.

976 **Fig. 3: HIF1 $\alpha$ -AS1 TFR2 RNA forms *in vitro* DNA:DNA:RNA triplexes with the predicted DNA target**

977 **region in EPHA2.** **a**, <sup>1</sup>H-1D NMR spectra of the EPHA2 DNA duplex (black), HIF1 $\alpha$ -AS1 TFR2 RNA  
978 (blue), heteroduplex (dark grey) and EPHA2:HIF1 $\alpha$ -AS1-TFR2 triplex (red) in a temperature range  
979 between 288-308 K. **b**, Electromobility shift assay of EPHA2 ssDNA or dsDNA (ss or ds) alone or the  
980 dsDNA in combination with HIF1 $\alpha$ -AS1-TFR2. Two different RNA dosages (50 or 250 pmol) and three  
981 different HIF1 $\alpha$ -AS1-TFR2 RNA lengths (27 nt, 46 nt, 131 nt) were used **c**, Circular dichroism spectra  
982 of the EPHA2 DNA duplex (black), the heteroduplex (dark grey) and EPHA2:HIF1 $\alpha$ -AS1-TFR2 triplex

983 (red) measured at 298 K. **d**, UV melting assay of the EPHA2 DNA duplex (black), the heteroduplex  
984 (dark grey) and EPHA2:HIF1 $\alpha$ -AS1-TFR2 triplex (red).

985 **Fig. 4: HIF1 $\alpha$ -AS1 limits EPHA2 and ADM expression through TFR2.** **a&b**, CRISPRa (a, n=6) or CRISPRi  
986 (b, n=3) targeting HIF1 $\alpha$ -AS1 in HUVECs followed by RT-qPCR for HIF1 $\alpha$ -AS1, EPHA2 and ADM. n=6,  
987 Paired t-test. **c**, Western blot with (AS1) or without (- and CTL) LNA GapmeR-mediated knockdown of  
988 HIF1 $\alpha$ -AS1 in two different batches of HUVEC. GAPDH was used as loading control. M, marker. **d**,  
989 Spheroid outgrowth assay of HUVECs treated with or without siRNAs against EPHA2. Cells treated  
990 under basal or VEGF-A (1 ng/mL) conditions for 16 h are shown. **e**, Quantification of the cumulative  
991 sprout length from the spheroid assay seen in Fig. 4d. One-Way ANOVA with Bonferroni post hoc  
992 test. n=12-15. **f**, Spheroid outgrowth assay of HUVECs treated with LNA GapmeRs targeting HIF1 $\alpha$ -  
993 AS1. Cells treated under basal, VEGF-A (1 ng/mL) or bFGF (3 ng/mL) conditions for 16 h are shown.  
994 LNA CTL served as negative control. Scale bar indicates 200  $\mu$ m. **g**, Quantification of the cumulative  
995 sprout length from the spheroid outgrowth assay seen in Fig. 4f. One-Way ANOVA with Bonferroni  
996 post hoc test. n=12-32. **h**, Scheme of the CRISPR Arcitect approach. TFR2 of HIF1 $\alpha$ -AS1 (underlined)  
997 was targeted with Cas9/gRNA and replaced with ssODNs including MEG3 TFR or a DNA fragment of  
998 luciferase negative control. **i-k**, RT-qPCR of TGFBR1 (i), EPHA2 (j) or ADM (k) after replacement of  
999 HIF1 $\alpha$ -AS1-TFR2 with MEG3-TFR or a DNA fragment of a luciferase negative control. NC, nontemplate  
1000 control. n=5, Paired t-test. Error bars are defined as mean +/- SEM. \*p<0.05. AS1, HIF1 $\alpha$ -AS1.

1001 **Fig. 5: HIF1 $\alpha$ -AS1 interacts directly with the HUSH complex member MPP8.** **a**, Volcano plot of  
1002 HIF1 $\alpha$ -AS1 protein interaction partners after RNA pulldown assay and ESI-MS/MS measurements  
1003 with fold enrichment and p-value. n=5. Proteins above the line (p<0.05) indicate significantly  
1004 associated proteins. **b**, List of proteins enriched after RNA pulldown assay, their p-value and fold  
1005 change. **c**, RIP with MPP8 antibodies and qPCR for HIF1 $\alpha$ -AS1 TFR2. IgG served as negative control.  
1006 n=4, Mann Whitney t-test. **d**, RIP with histone3-lysine9-trimethylation antibodies and qPCR for  
1007 HIF1 $\alpha$ -AS1 TFR2. IgG served as negative control. n=3, One-Way ANOVA with Dunnett's post hoc test.  
1008 **e**, Scheme of the different HIF1 $\alpha$ -AS1 RNAs used for *in vitro* RNA immunoprecipitation. **f**, RT-qPCR  
1009 after *in vitro* binding assay of purified MPP8 with *in vitro* transcribed HIF1 $\alpha$ -AS1 RNAs. MPP8  
1010 antibodies were used for RNA immunoprecipitation. An T7-MCS *in vitro* transcribed RNA served as  
1011 negative control (CTL). FL, full length; E1, Exon1; E2, Exon2.  $\Delta$  indicates the deleted nt from HIF1 $\alpha$ -  
1012 AS1 full length. **g-h**, Proximity ligation assay of HUVECs with antibodies against MPP8 and H3K9me3  
1013 (g) or MPP8 and SETDB1 (h). The individual antibody alone served as negative control. Red dots  
1014 indicate polymerase amplified interaction signals. Scale bar indicates 20  $\mu$ m (g) or 10  $\mu$ m (h). Error  
1015 bars are defined as mean +/- SEM. \*p<0.05.

1016 **Fig. 6: HIF1 $\alpha$ -AS1 directs the HUSH complex member MPP8 and SETDB1 to triplex target sites.** **a**,  
1017 Chromatin immunoprecipitation (ChIP) with MPP8 antibodies with or without RNase A treatment and  
1018 qPCR for the triplex target sites of EPHA2 and ADM. Primers against a promoter sequence of GAPDH  
1019 served as negative control. n=4, paired t-test. **b-c**, ChIP with antibodies against SETDB1, MPP8 or  
1020 NP220 in HUVECs treated with (AS1) or without (CTL) LNA GapmeRs against HIF1 $\alpha$ -AS1. QPCR was  
1021 performed for EPHA2 TTS (b) or ADM TTS (c). n=5, paired t-test. **d**, IGV original traces loaded of ATAC-  
1022 Seq in HUVECs separately and as an overlay after knockdown of HIF1 $\alpha$ -AS1 (black), SETDB1 (green),  
1023 MPP8 (blue) or the negative control (pink). ChIP-Seq data (H3K4me3, H3K27Ac, H3K9Ac) in HUVECs  
1024 was derived from Encode. Numbers in square brackets indicate data range values. Red arrows  
1025 indicate altered chromatin accessible regions after knockdown. Error bars are defined as mean +/-  
1026 SEM. \*p<0.05.

1027 **Supplementary information**

1028 **Extended data figure 1: a**, Cumulative fold enrichment of the four remaining candidates in the U2OS  
1029 and HeLa S3 Triplex-Seq. **b**, RT-qPCR of HIF1 $\alpha$ -AS1 in paSMCs treated under hypoxic conditions (HOX,  
1030 1% O<sub>2</sub>) for 24 h. Cells treated under normoxia (NOX) served as basal control. n=4, Unpaired t-test. **c**,  
1031 RT-qPCR of HIF1 $\alpha$ -AS1 from endothelial cells isolated from glioblastoma (GBM) or adjacent healthy  
1032 control (CTL) tissue. n=5. Paired t-test. **d**, RT-qPCR of HIF1 $\alpha$ -AS1 in paSMCs from control donors  
1033 (Donor) or patients with idiopathic pulmonary arterial hypertension (IPAH). n=3, Unpaired t-test. **e**,  
1034 RT-qPCR of HIF1 $\alpha$ -AS1 after knockdown with LNA-GapmeRs against HIF1 $\alpha$ -AS1 or an LNA negative  
1035 control (CTL). n=4, Paired t-test. **f**, Agarose gel after RT-PCR of Exon1 (E1), Exon2 (E2) or the first  
1036 714nt of the pre-processed HIF1 $\alpha$ -AS1 (E1-I). Error bars are defined as mean +/- SEM. \*p<0.05.

1037 **Extended data figure 2: a**, <sup>1</sup>H-1D NMR spectra of the EPHA2\_CTGA hairpin (grey) and the  
1038 EPHA2\_CTGA:HIF1 $\alpha$ -AS1-TFR2 triplex (dark red) in a temperature range between 278-308 K. **b**, <sup>1</sup>H-1D  
1039 NMR spectra of the ADM\_CTGA hairpin (grey) and the ADM\_CTGA:HIF1 $\alpha$ -AS1-TFR2 triplex (dark red)  
1040 in a temperature range between 278-308 K. **c**, Circular dichroism spectra of the EPHA2:HIF1 $\alpha$ -AS1-  
1041 TFR2 (TFO2-23) triplex (red), the EPHA2\_CTGA hairpin alone (light grey) and the EPHA2\_CTGA:HIF1 $\alpha$ -  
1042 AS1-TFR2 (TFO2-23) triplex (dark red) measured at 298 K. **d**, UV melting of the EPHA2:HIF1 $\alpha$ -AS1-  
1043 TFR2 (TFO2-23) triplex (red), the EPHA2\_CTGA hairpin (light grey) and EPHA2\_CTGA:HIF1 $\alpha$ -AS1-TFR2  
1044 (TFO2-23) (dark red). **e**, Circular dichroism spectra of the the ADM duplex (black), the heteroduplex  
1045 (dark grey), the ADM\_CTGA hairpin alone (light grey) and the ADM\_CTGA:HIF1 $\alpha$ -AS1-TFR2 (TFO2-23)  
1046 triplex (dark red) measured at 298 K. **f**, UV melting of the ADM duplex (black), the heteroduplex (dark  
1047 grey), the ADM\_CTGA hairpin (light grey) and ADM\_CTGA:HIF1 $\alpha$ -AS1-TFR2 (TFO2-23) triplex (dark  
1048 red).

1049 **Extended data figure 3: a**, RT-qPCR after siRNA-mediated knockdown of EPHA2. Expression levels of  
1050 EPHA2 are shown. Scrambled siRNA (CTL) served as negative control. n=3, Unpaired t-test. **b**,  
1051 Western blot with (si) or without (CTL) siRNA-mediated knockdown of EPHA2 in three different  
1052 batches of HUVEC. EPHA2 and HSC70/HSP70 antibodies were used. M, marker. **c**, Quantification of  
1053 the sprout numbers from the spheroid assay seen in Fig. 4d. One-Way ANOVA with Bonferroni post  
1054 hoc test. n=12-15. **d**, Quantification of the sprout numbers from the spheroid assay seen in Fig. 4f.  
1055 One-Way ANOVA with Bonferroni post hoc test. n=12-32. **e**, Relative RNA level of HIF1 $\alpha$ -AS1 TFR2  
1056 after a ssODN-mediated replacement of the TFR2 within HIF1 $\alpha$ -AS1 with the TFR of MEG3 or a DNA  
1057 fragment of a luciferase negative control. NC, nontemplate control. n=5, Paired t-test. Error bars are  
1058 defined as mean +/- SEM. \*p<0.05.

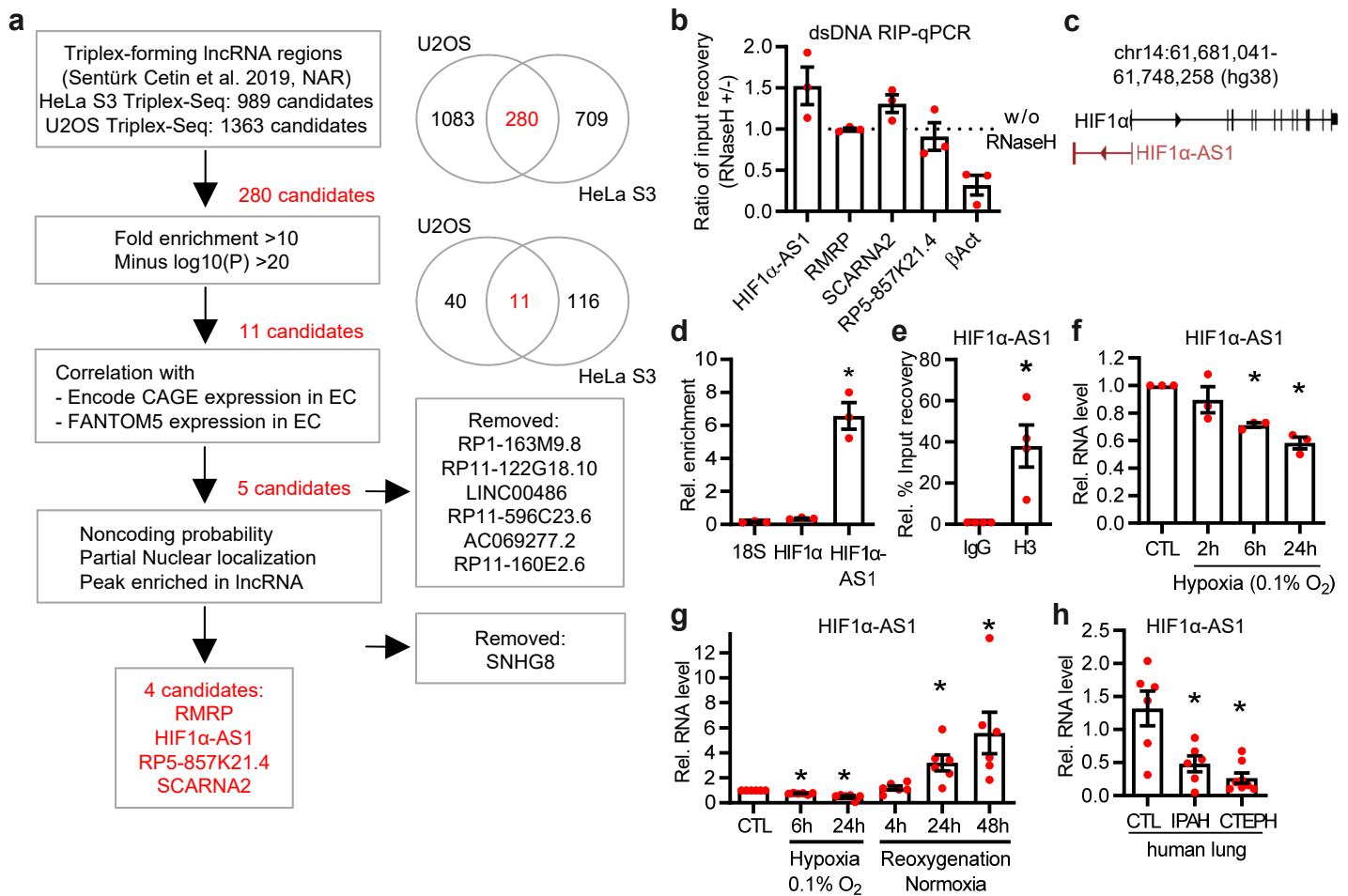
1059 **Extended data figure 4: a&b**, RIP with MPP8 antibodies and qPCR for HIF1 $\alpha$ -AS1 (a) or HIF1 $\alpha$  (b). IgG  
1060 served as negative control. n=4, Mann Whitney t-test. **c**, Binding propensity of MPP8 and HIF1 $\alpha$ -AS1  
1061 calculated with *catRAPID*. **d**, Proximity ligation assay of HUVECs with antibodies against MPP8 and  
1062 dsDNA. The individual antibody alone served as negative control. Red dots indicate polymerase  
1063 amplified interaction signals. Scale bar indicates 20  $\mu$ m. Error bars are defined as mean +/- SEM.  
1064 \*p<0.05.

1065 **Sup. Table 1:** Triplex-Seq HeLa S3 lncRNA regions

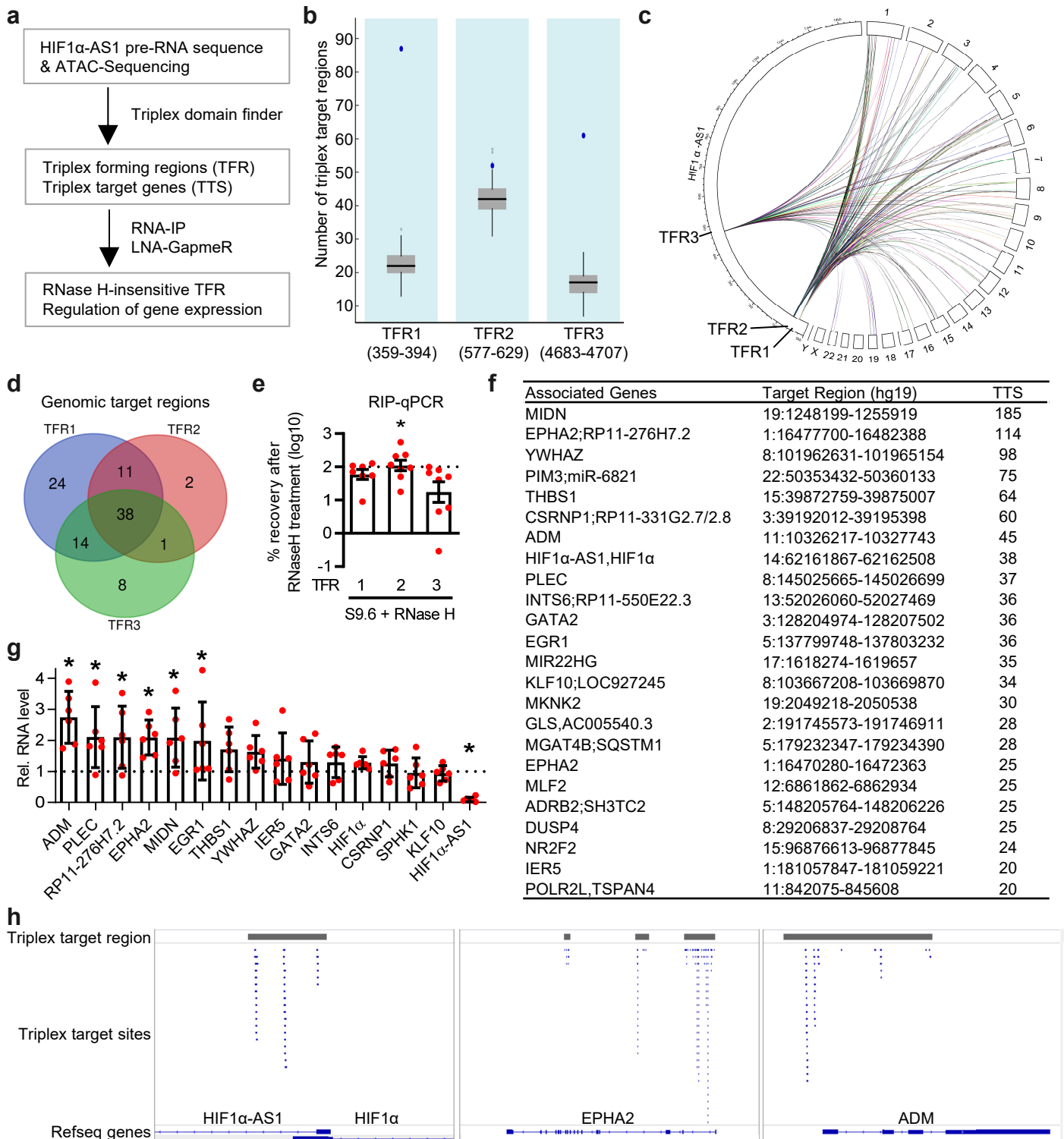
1066 **Sup. Table 2:** Triplex-Seq U2OS lncRNA regions

1067 **Sup. Table 3:** List of TTS of TFR1

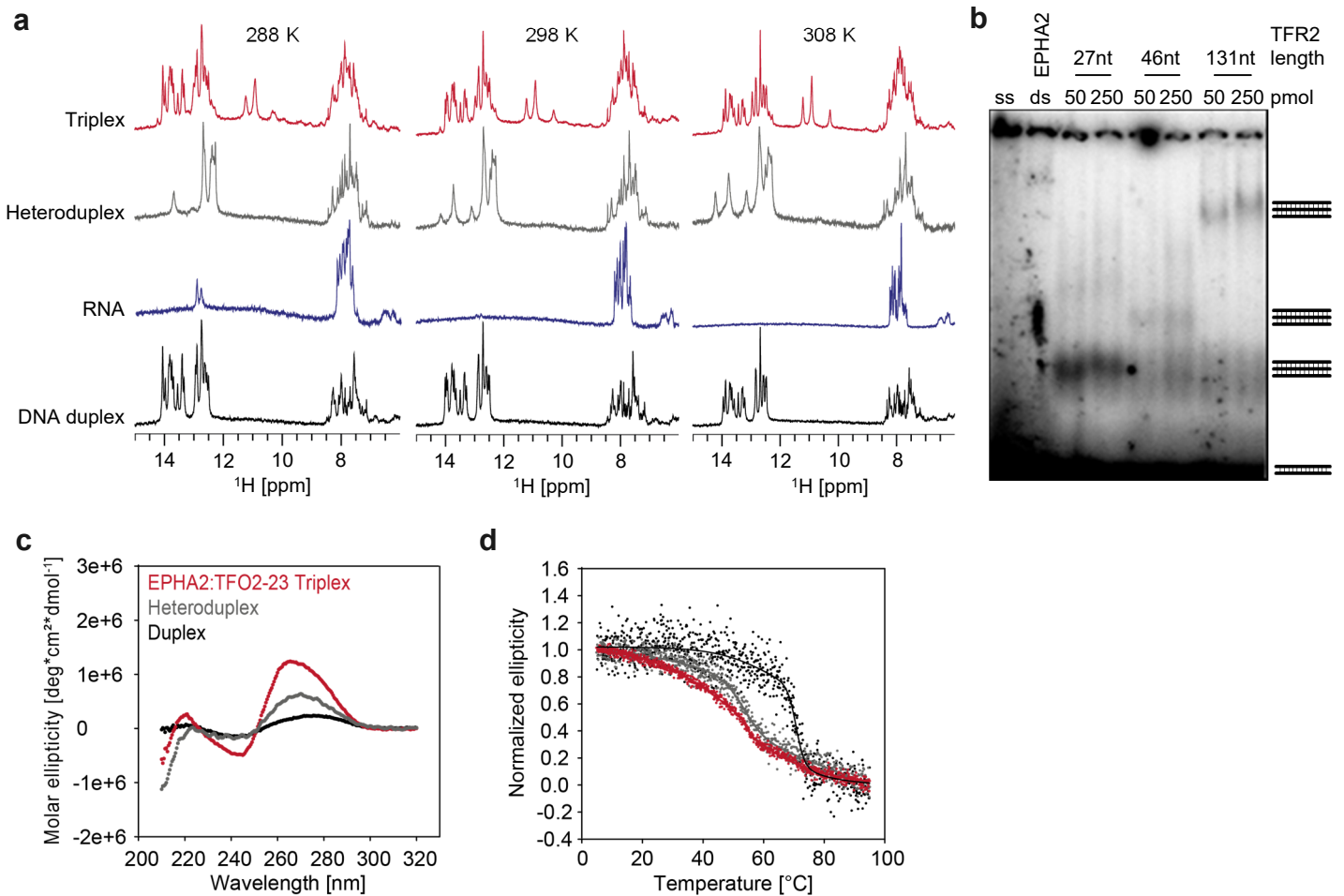
- 1068 **Sup. Table 4:** List of TTS of TFR2
- 1069 **Sup. Table 5:** List of TTS of TFR3
- 1070 **Sup. Table 6:** Interaction partners of HIF1 $\alpha$ -AS1



**Fig. 1: HIF1α-AS1 is a triplex- and DNA-associated RNase H-insensitive lncRNA in endothelial cells.** **a**, Overview of the identification of endothelial-expressed triplex-forming lncRNAs. lncRNAs from a previous Triplex-Seq study in HeLa S3 and U2OS were overlapped, filtered with high stringency and analyzed for nuclear expression in endothelial cells with Encode and FANTOM5 CAGE data followed by analyses for noncoding probability and enriched peaks in the Triplex-Seq data. **b**, RNA-immunoprecipitation with anti-dsDNA followed by qPCR (RIP-qPCR) targeting the lncRNA candidates in HUVEC. Samples were treated with or without RNase H. βAct served as control for RNase H-mediated degradation. n=3. **c**, Scheme of the human genomic locus of HIF1α-AS1. **d**, RT-qPCR after anti-dsDNA-RIP in HUVEC. HIF1α and 18S rRNA served as negative control. One-way ANOVA with Tukey's post hoc test, n=3. **e**, RIP-qPCR with anti-histone3 (H3) in HUVEC. Data was normalized against GAPDH. Paired t-test, n=4. **f**, RT-qPCR of HIF1α-AS1 in HUVEC treated with hypoxia (0.1% O<sub>2</sub>) for the indicated time points. Normoxia served as negative control (CTL). n=3, One-Way ANOVA with Bonferroni post hoc test. **g**, RT-qPCR of HIF1α-AS1 in HUVECs treated with hypoxia (0.1% O<sub>2</sub>) followed by reoxygenation with normoxia (after 24 h hypoxia) for the indicated time points. n=6, One-Way ANOVA with Dunnett's post hoc test. **h**, RT-qPCR of HIF1α-AS1 in lungs from control donors (CTL, n=6) or patients with idiopathic pulmonary arterial hypertension (IPAH, n=6) or chronic thromboembolic pulmonary hypertension (CTEPH, n=8). One-Way ANOVA with Tukey's post hoc test. Error bars are defined as mean +/- SEM. \*p<0.05.

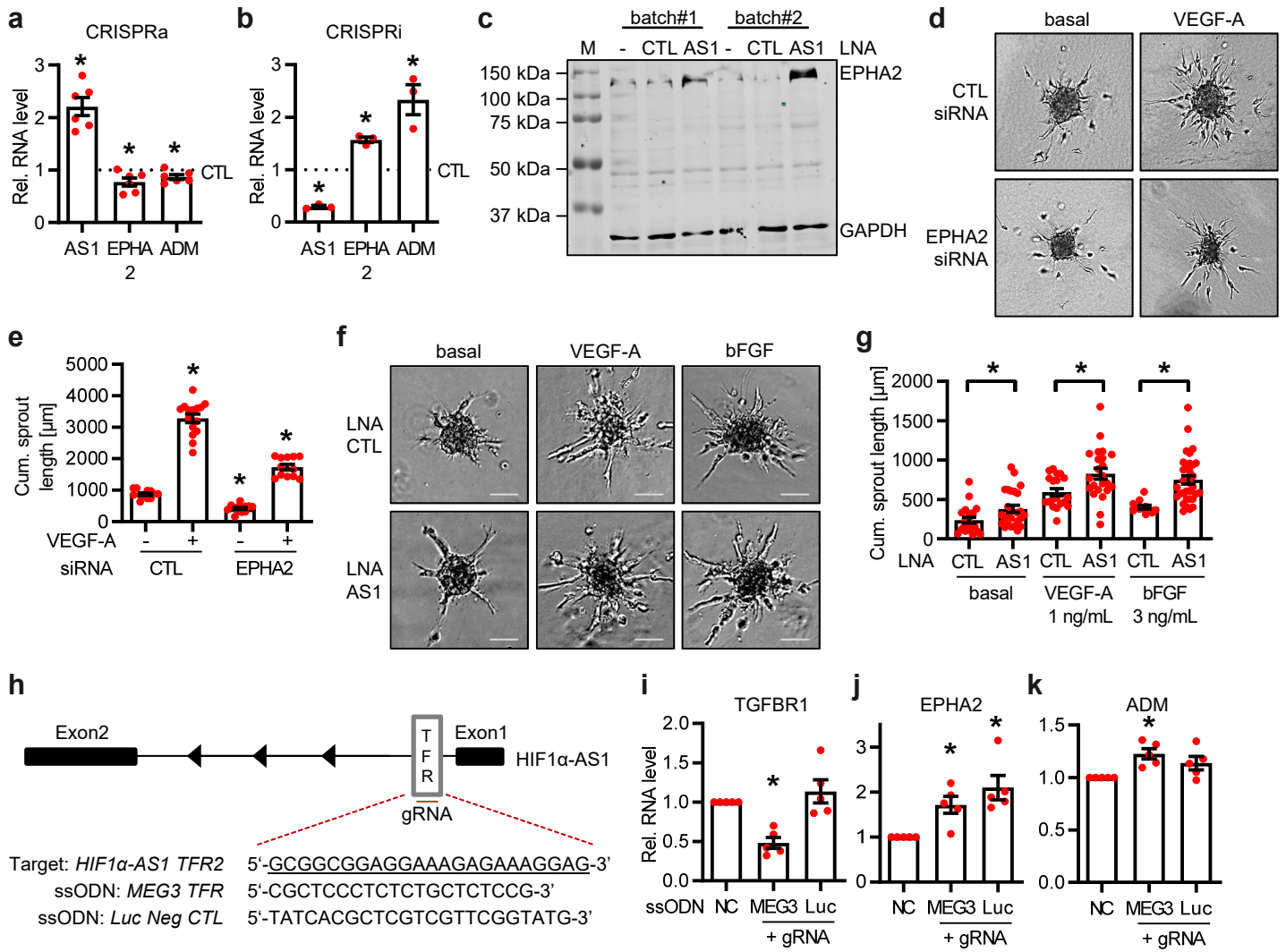


**Fig. 2: HIF1α-AS1 potentially forms DNA:DNA:RNA triplexes.** **a**, Overview of the identification of HIF1α-AS1 triplex forming regions (TFR) and their DNA triplex target sites (TTS) with triplex domain finder (TDF). HIF1α-AS1 pre-RNA and ATAC-Seq of HUVECs treated with or without LNA GapmeRs against HIF1α-AS1 were used as input. RIP and LNA GapmeRs were used to validate the findings obtained by TDF. **b**, Number of triplex target regions of three statistically significant TFRs of HIF1α-AS1 identified with TDF. Numbers in brackets represent the position of the individual TFR within HIF1α-AS1 pre-RNA. All TFRs have a significantly higher number of triplex target regions in targets (blue) than non-target regions (grey). **c**, Circos plot showing the localization of the individual TFR within HIF1α-AS1 pre-RNA and its interaction with the chromosomal TTS. **d**, Overlap of TTS of the individual TFRs of HIF1α-AS1. **e**, Identification of RNase H-resistant TFRs. RIP with S9.6 RNA/DNA hybrid antibody with or without RNase H treatment in HUVEC followed by qPCR for the TFRs. Ratio of %-input recovery with/without RNase H treatment is shown. n=8, paired t-test. **f**, HIF1α-AS1 TFR2 top target genes, their genomic location and number of TTS identified by TDF. **g**, RT-qPCR of triplex target genes of TFR2 after knockdown of HIF1α-AS1 in HUVEC. n=6, One-Way ANOVA with Holm's Sidak post hoc test. **h**, Three different triplex target regions of HIF1α-AS1 are shown. Triplex target regions are highlighted in grey, triplex target sites are shown in blue. Error bars are defined as mean +/- SEM. \*p<0.05.

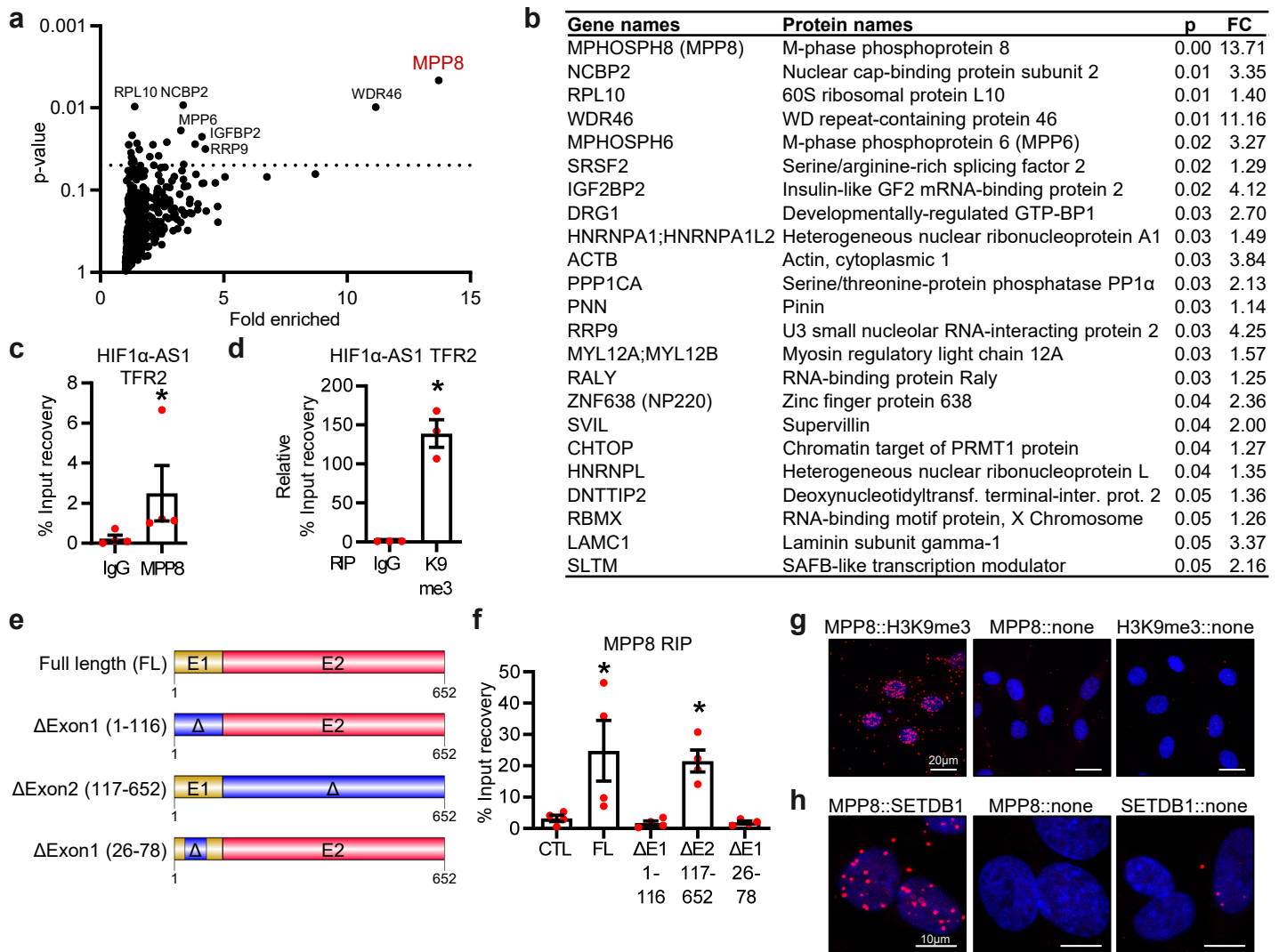


**Fig. 3: HIF1 $\alpha$ -AS1 TFR2 RNA forms *in vitro* DNA:DNA:RNA triplexes with the predicted DNA target region in EPHA2. a,**  $^1\text{H}$ -1D NMR spectra of the EPHA2 DNA duplex (black), HIF1 $\alpha$ -AS1 TFR2 RNA (blue), heteroduplex (dark grey) and EPHA2:HIF1 $\alpha$ -AS1-TFR2 triplex (red) in a temperature range between 288-308 K. **b,** Electromobility shift assay of EPHA2 ssDNA or dsDNA (ss or ds) alone or the dsDNA in combination with HIF1 $\alpha$ -AS1-TFR2. Two different RNA dosages (50 or 250 pmol) and three different HIF1 $\alpha$ -AS1-TFR2 RNA lengths (27 nt, 46 nt, 131 nt) were used. **c,** Circular dichroism spectra of the EPHA2 DNA duplex (black), the heteroduplex (dark grey) and EPHA2:HIF1 $\alpha$ -AS1-TFR2 triplex (red) measured at 298 K. **d,** UV melting assay of the EPHA2 DNA duplex (black), the heteroduplex (dark grey) and EPHA2:HIF1 $\alpha$ -AS1-TFR2 triplex (red).

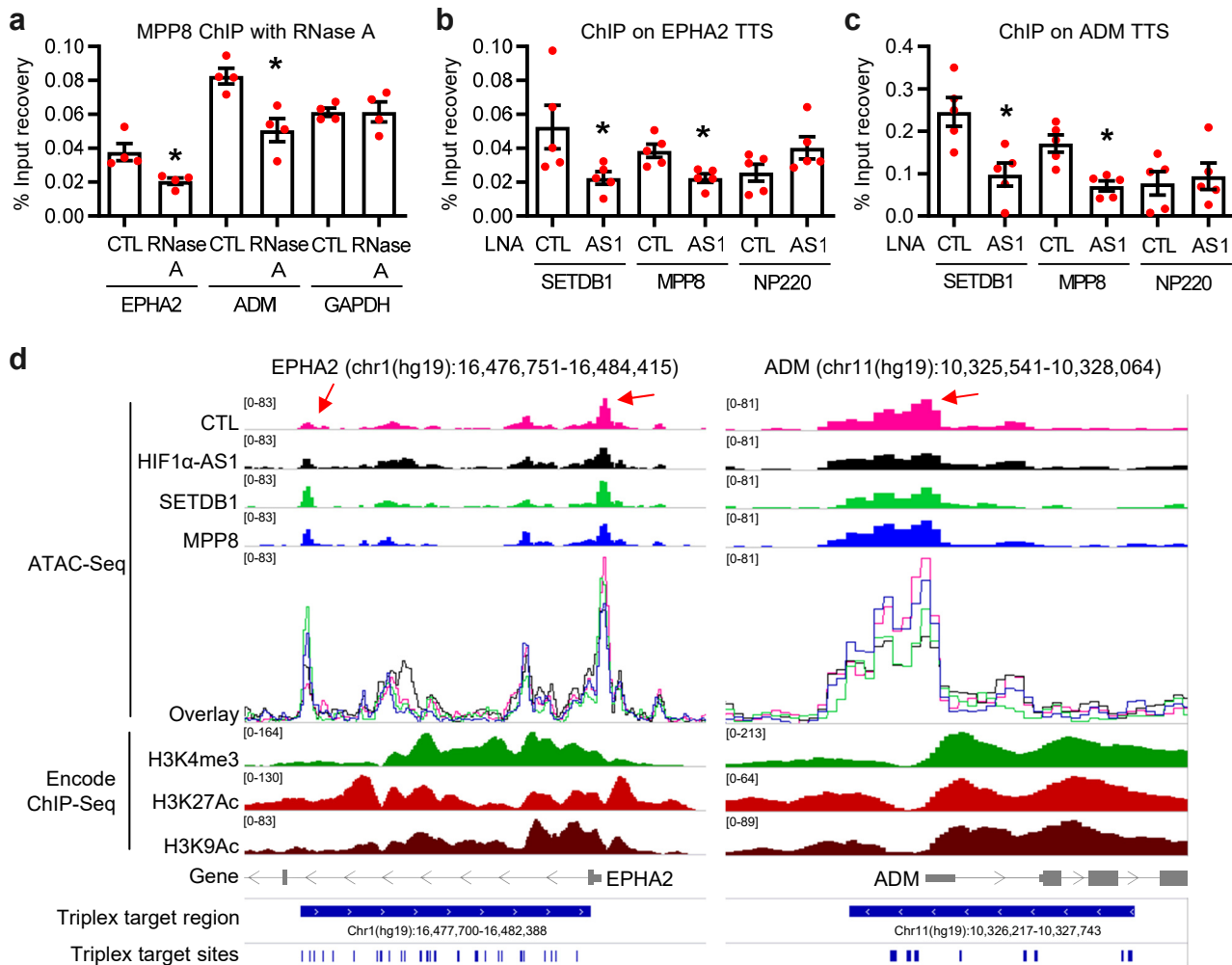




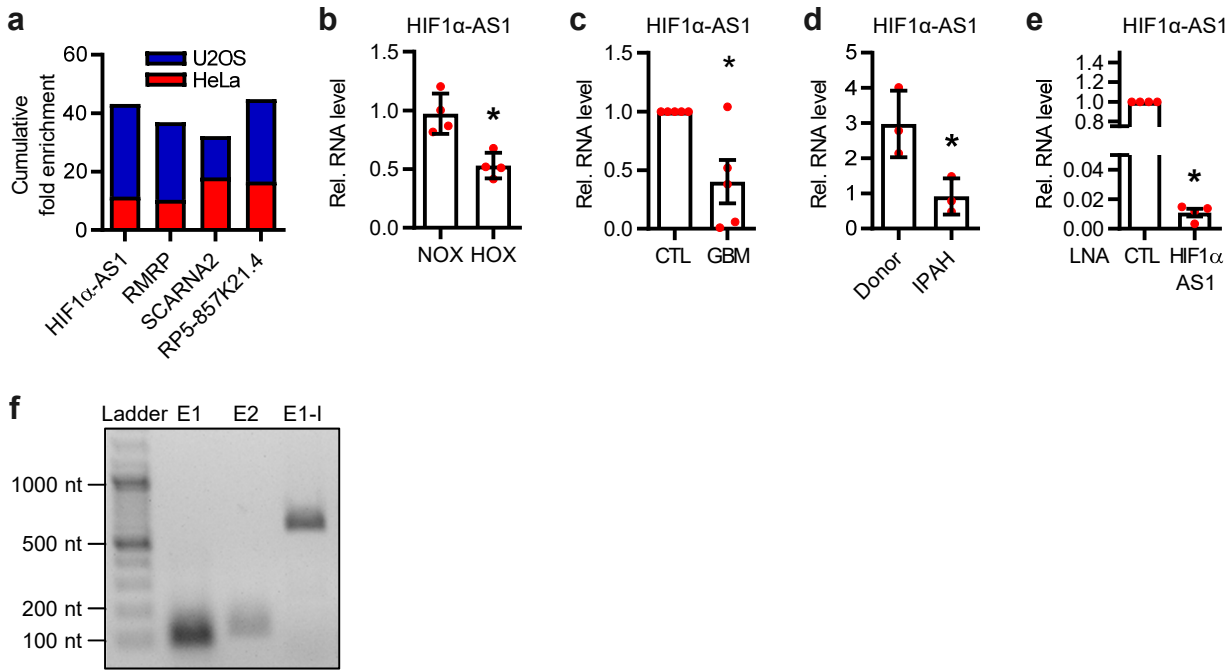
**Fig. 4: HIF1α-AS1 limits EPHA2 and ADM expression through TFR2.** **a&b**, CRISPRa (a, n=6) or CRISPRi (b, n=3) targeting HIF1α-AS1 in HUVECs followed by RT-qPCR for HIF1α-AS1, EPHA2 and ADM. n=6, Paired t-test. **c**, Western blot with (AS1) or without (- and CTL) LNA GapmeR-mediated knockdown of HIF1α-AS1 in two different batches of HUVEC. GAPDH was used as loading control. M, marker. **d**, Spheroid outgrowth assay of HUVECs treated with or without siRNAs against EPHA2. Cells treated under basal or VEGF-A (1 ng/mL) conditions for 16 h are shown. **e**, Quantification of the cumulative sprout length from the spheroid assay seen in Fig. 4d. One-Way ANOVA with Bonferroni post hoc test. n=12-15. **f**, Spheroid outgrowth assay of HUVECs treated with LNA GapmeRs targeting HIF1α-AS1. Cells treated under basal, VEGF-A (1 ng/mL) or bFGF (3 ng/mL) conditions for 16 h are shown. LNA CTL served as negative control. Scale bar indicates 200 μm. **g**, Quantification of the cumulative sprout length from the spheroid outgrowth assay seen in Fig. 4f. One-Way ANOVA with Bonferroni post hoc test. n=12-32. **h**, Scheme of the CRISPR/Cas9 approach. TFR2 of HIF1α-AS1 (underlined) was targeted with Cas9/gRNA and replaced with ssODNs including MEG3-TFR or a DNA fragment of luciferase negative control. **i-k**, RT-qPCR of TGFBR1 (i), EPHA2 (j) or ADM (k) after replacement of HIF1α-AS1-TFR2 with MEG3-TFR or a DNA fragment of a luciferase negative control. NC, nontemplate control. n=5, Paired t-test. Error bars are defined as mean ± SEM. \*p<0.05. AS1, HIF1α-AS1.



**Fig. 5: HIF1α-AS1 interacts directly with the HUSH complex member MPP8.** **a**, Volcano plot of HIF1α-AS1 protein interaction partners after RNA pulldown assay and ESI-MS/MS measurements with fold enrichment and p-value.  $n=5$ . Proteins above the line ( $p<0.05$ ) indicate significantly associated proteins. **b**, List of proteins enriched after RNA pulldown assay, their p-value and fold change. **c**, RIP with MPP8 antibodies and qPCR for HIF1α-AS1 TFR2. IgG served as negative control.  $n=4$ , Mann Whitney t-test. **d**, RIP with histone3-lysine9-trimethylation antibodies and qPCR for HIF1α-AS1 TFR2. IgG served as negative control.  $n=3$ , One-Way ANOVA with Dunnett's post hoc test. **e**, Scheme of the different HIF1α-AS1 RNAs used for *in vitro* RNA immunoprecipitation. **f**, RT-qPCR after *in vitro* binding assay of purified MPP8 with *in vitro* transcribed HIF1α-AS1 RNAs. MPP8 antibodies were used for RNA immunoprecipitation. An T7-MCS *in vitro* transcribed RNA served as negative control (CTL). FL, full length; E1, Exon1; E2, Exon2. Δ indicates the deleted nt from HIF1α-AS1 full length. **g-h**, Proximity ligation assay of HUVECs with antibodies against MPP8 and H3K9me3 (**g**) or MPP8 and SETDB1 (**h**). The individual antibody alone served as negative control. Red dots indicate polymerase amplified interaction signals. Scale bar indicates 20 μm (**g**) or 10 μm (**h**). Error bars are defined as mean  $\pm$  SEM. \* $p<0.05$ .

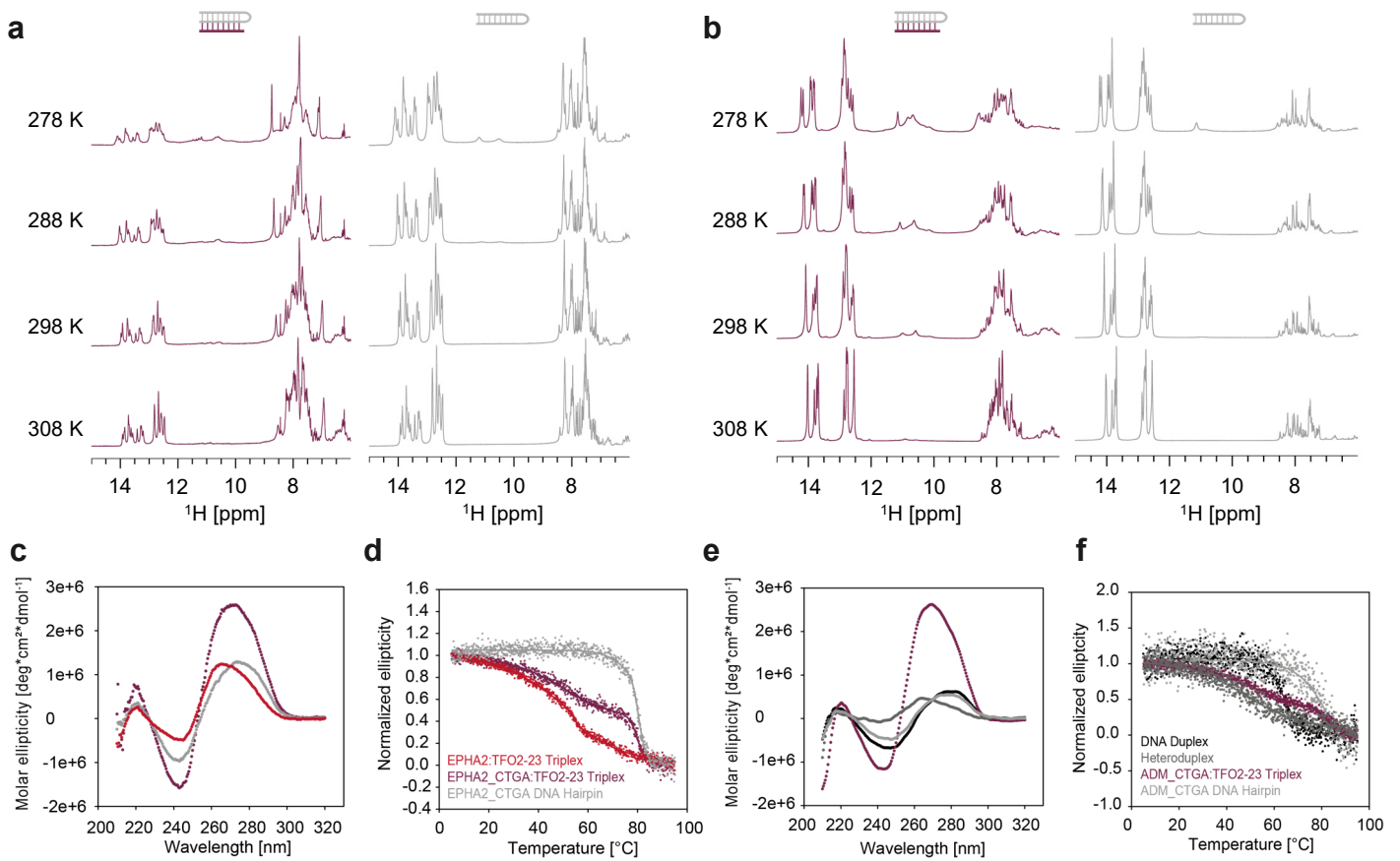


**Fig. 6: HIF1 $\alpha$ -AS1 directs the HUSH complex member MPP8 and SETDB1 to triplex target sites.** **a**, Chromatin immunoprecipitation (ChIP) with MPP8 antibodies with or without RNase A treatment and qPCR for the triplex target sites of EPHA2 and ADM. Primers against a promoter sequence of GAPDH served as negative control. n=4, paired t-test. **b-c**, ChIP with antibodies against SETDB1, MPP8 or NP220 in HUVECs treated with (AS1) or without (CTL) LNA GapmeRs against HIF1 $\alpha$ -AS1. QPCR was performed for EPHA2 TTS (**b**) or ADM TTS (**c**). n=5, paired t-test. **d**, IGV original traces loaded of ATAC-Seq in HUVECs separately and as an overlay after knockdown of HIF1 $\alpha$ -AS1 (black), SETDB1 (green), MPP8 (blue) or the negative control (pink). ChIP-Seq data (H3K4me3, H3K27Ac, H3K9Ac) in HUVECs was derived from Encode. Numbers in square brackets indicate data range values. Red arrows indicate altered chromatin accessible regions after knockdown. Error bars are defined as mean  $\pm$  SEM. \*p<0.05.



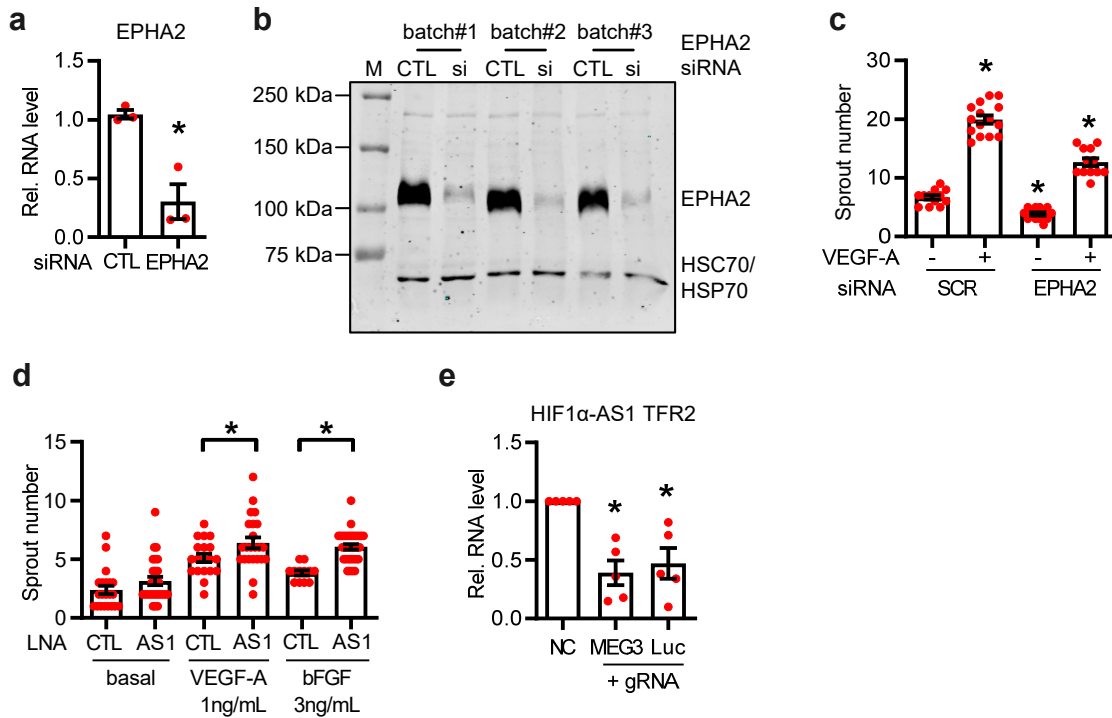
**Extended data figure 1:**

**a**, Cumulative fold enrichment of the four remaining candidates in the U2OS and HeLa S3 Triplex-Seq. **b**, RT-qPCR of HIF1α-AS1 in paSMCs treated under hypoxic conditions (HOX, 1% O<sub>2</sub>) for 24 h. Cells treated under normoxia (NOX) served as basal control. n=4, Unpaired t-test. **c**, RT-qPCR of HIF1α-AS1 from endothelial cells isolated from glioblastoma (GBM) or adjacent healthy control (CTL) tissue. n=5. Paired t-test. **d**, RT-qPCR of HIF1α-AS1 in paSMCs from control donors (Donor) or patients with idiopathic pulmonary arterial hypertension (IPAH). n=3, Unpaired t-test. **e**, RT-qPCR of HIF1α-AS1 after knockdown with LNA-GapmeRs against HIF1α-AS1 or an LNA negative control (CTL). n=4, Paired t-test. **f**, Agarose gel after RT-PCR of Exon1 (E1), Exon2 (E2) or the first 714nt of the pre-processed HIF1α-AS1 (E1-I). Error bars are defined as mean +/- SEM. \*p<0.05.



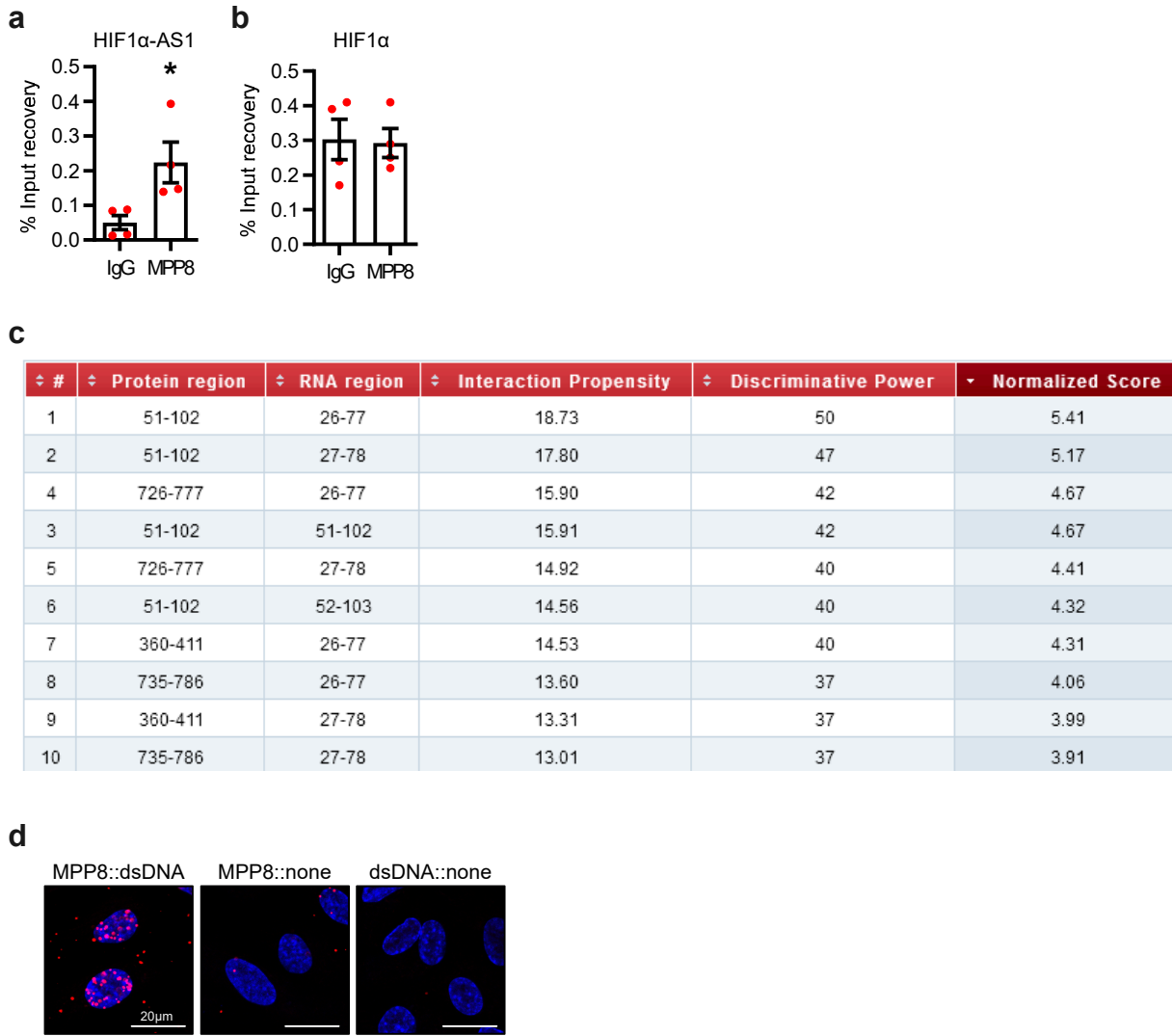
**Extended data figure 2:**

**a**, <sup>1</sup>H-1D NMR spectra of the EPHA2\_CTGA hairpin (grey) and the EPHA2\_CTGA:HIF1 $\alpha$ -AS1-TFR2 triplex (dark red) in a temperature range between 278-308 K. **b**, <sup>1</sup>H-1D NMR spectra of the ADM\_CTGA hairpin (grey) and the ADM\_CTGA:HIF1 $\alpha$ -AS1-TFR2 triplex (dark red) in a temperature range between 278-308 K. **c**, Circular dichroism spectra of the EPHA2:HIF1 $\alpha$ -AS1-TFR2 (TFO2-23) triplex (red), the EPHA2\_CTGA hairpin alone (light grey) and the EPHA2\_CTGA:HIF1 $\alpha$ -AS1-TFR2 (TFO2-23) triplex (dark red) measured at 298 K. **d**, UV melting of the EPHA2:HIF1 $\alpha$ -AS1-TFR2 (TFO2-23) triplex (red), the EPHA2\_CTGA hairpin (light grey) and EPHA2\_CTGA:HIF1 $\alpha$ -AS1-TFR2 (TFO2-23) (dark red). **e**, Circular dichroism spectra of the the ADM duplex (black), the heteroduplex (dark grey), the ADM\_CTGA hairpin alone (light grey) and the ADM\_CTGA:HIF1 $\alpha$ -AS1-TFR2 (TFO2-23) triplex (dark red) measured at 298 K. **f**, UV melting of the ADM duplex (black), the heteroduplex (dark grey), the ADM\_CTGA hairpin (light grey) and ADM\_CTGA:HIF1 $\alpha$ -AS1-TFR2 (TFO2-23) triplex (dark red).



**Extended data figure 3:**

**a**, RT-qPCR after siRNA-mediated knockdown of EPHA2. Expression levels of EPHA2 are shown. Scrambled siRNA (CTL) served as negative control. n=3, Unpaired t-test. **b**, Western blot with (si) or without (CTL) siRNA-mediated knockdown of EPHA2 in three different batches of HUVEC. EPHA2 and HSC70/HSP70 antibodies were used. M, marker **c**, Quantification of the sprout numbers from the spheroid assay seen in Fig. 4d. One-Way ANOVA with Bonferroni post hoc test. n=12-15. **d**, Quantification of the sprout numbers from the spheroid assay seen in Fig. 4f. One-Way ANOVA with Bonferroni post hoc test. n=12-32. **e**, Relative RNA level of HIF1α-AS1 TFR2 after a ssODN-mediated replacement of the TFR2 within HIF1α-AS1 with the TFR of MEG3 or a DNA fragment of a luciferase negative control. NC, nontemplate control. n=5, Paired t-test. Error bars are defined as mean +/- SEM. \*p<0.05.



**Extended data figure 4:**

**a&b**, RIP with MPP8 antibodies and qPCR for HIF1 $\alpha$ -AS1 (a) or HIF1 $\alpha$  (b). IgG served as negative control. n=4, Mann Whitney t-test. **c**, Binding propensity of MPP8 and HIF1 $\alpha$ -AS1 calculated with *catRAPID*. **d**, Proximity ligation assay of HUVECs with antibodies against MPP8 and dsDNA. The individual antibody alone served as negative control. Red dots indicate polymerase amplified interaction signals. Scale bar indicates 20  $\mu$ m. Error bars are defined as mean  $\pm$  SEM. \*p<0.05.