An Intrinsic Flk1 Enhancer Directs Arterial-Specific Expression via RBPJ-Mediated Venous Repression

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Objective—The vascular endothelial growth factor (VEGF) receptor Flk1 is essential for vascular development, but the signaling and transcriptional pathways by which its expression is regulated in endothelial cells remain unclear. Although previous studies have identified 2 Flk1 regulatory enhancers, these are dispensable for Flk1 expression, indicating that additional enhancers contribute to Flk1 regulation in endothelial cells. In the present study, we sought to identify Flk1 enhancers contributing to expression in endothelial cells.

Approach and Results—A region of the 10th intron of the Flk1 gene (Flk1in10) was identified as a putative enhancer and tested in mouse and zebrafish transgenic models. This region robustly directed reporter gene expression in arterial endothelial cells. Using a combination of targeted mutagenesis of transcription factor–binding sites and gene silencing of transcription factors, we found that Gata and Ets factors are required for Flk1in10 enhancer activity in all endothelial cells. Furthermore, we showed that activity of the Flk1in10 enhancer is restricted to arteries through repression of gene expression in venous endothelial cells by the Notch pathway transcriptional regulator Rbpj.

Conclusions—This study demonstrates a novel mechanism of arterial–venous identity acquisition, indicates a direct link between the Notch and VEGF signaling pathways, and illustrates how cis-regulatory diversity permits differential expression outcomes from a limited repertoire of transcriptional regulators. (Arterioscler Thromb Vasc Biol. 2016;36:1209-1219. DOI: 10.1161/ATVB.AHA.116.307517.)

Key Words: arterial-venous specification • artery • endothelial cells • mice • notch • veins • zebrafish
Nonstandard Abbreviations and Acronyms

| Abbreviation | Description                      |
|--------------|----------------------------------|
| E            | embryonic day                    |
| EMSA         | electrophoretic mobility shift assay |
| GFP          | green fluorescent protein        |
| NICD         | Notch intracellular domain       |
| VEGF         | vascular endothelial growth factor |

Conserved between the 2 model systems, the specific roles for each receptor may have diverged.11

Previous studies identified a pan-endothelial enhancer within the first intron of Flk1 (Flk1intron1)12; however, deletion of this enhancer had no detectable effect on Flk1 expression.13 A second Flk1 enhancer (Flk1 dorsal multipotent mesodermal enhancer), located 5’ upstream of Flk1,14 directs expression in the lateral mesoderm during early embryogenesis but not in differentiated endothelial cells.15 Consequently, it is likely that additional enhancer elements contribute to Flk1 activity in endothelial cells, similar to the multiple endothelial-specific enhancers regulating the Mef2c, Tal1, and Dll4 loci.4,12 These arrangements of enhancers, binding a varied array of transcription factors downstream of multiple signaling inputs, may explain the considerable phenotypic heterogeneity of endothelial cells among tissues, developmental stages, and angiogenic status.

Many different signaling cascades, including Wnt, transforming growth factor-β, and Notch, interact with the VEGF pathway at multiple stages of vascular development, yet these interactions are multifaceted, context dependent, and affect gene expression patterns both directly and indirectly.14-16 Given the crucial role of VEGFs signaling in both developmental and tumor angiogenesis, a more complete understanding of the manner in which components of the VEGF pathway are regulated is key to our understanding of how vascular signaling pathways interact to pattern the embryonic vasculature correctly, as well as our ability to modulate vessel growth accurately in pathological conditions.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
Identification of an Arterial-Restricted Endothelial Cell Enhancer Within the 10th Intron of the Flk1 Gene
An in silico search of the mouse and human Flk1/KDR loci for conserved sequences enriched in enhancer-associated histone modifications and endothelial cell–specific DNaseI hypersensitivity sites identified the 10th intron of Flk1 (Flk1in10) as a putative enhancer region (Figure I in the online-only Data Supplement). To verify and characterize the activity of this region, the 825-bp mouse Flk1in10 sequence was cloned upstream of the silent hsp68 minimal promoter and LacZ reporter gene and used to generate the stable transgenic LacZ line21 to enable analysis of GFP expression patterns within the vasculature (Figure 2B–2F). GFP expression was detected in the axial, intersegmental, and head vessels; blood cells; and the heart, suggesting that the transcriptional pathways regulating mouse Flk1in10 are conserved in zebrafish. At 48 hours post fertilization, reporter gene expression within the axial vessels diminished and was detected weakly only in the dorsal aorta. Furthermore, GFP expression became restricted to efferent intersegmental vessels (Figure 2D; Movie I in the online-only Data Supplement). Restriction of the transgene to the intersegmental arteries but not intersegmental veins was also clearly detected at 72 hours post fertilization, although expression was now mostly absent in the axial vessels (Figure 2E and 2F; Figure III in the online-only Data Supplement). To rule out a role for flow in the establishment of this expression pattern, transgene expression was investigated in embryos lacking heart function. Although the absence of flow resulted in a decrease in GFP intensity, arterial restriction in the intersegmental vessels was still observed (Figure IV in the online-only Data Supplement).

These results demonstrate that Flk1in10 represents a novel, arterial-restricted gene enhancer, unlike the Flk1 intron1 enhancer, which directs pan-vascular expression22 (Figure V in the online-only Data Supplement). This supports the hypothesis that regulation of Flk1 in the vasculature is achieved by a number of modular enhancer elements and suggests that VEGF receptor availability can be regulated independently in different endothelial subpopulations. Although endogenous mouse Flk1 mRNA is found in both arterial and venous endothelial cells during embryonic development,9 interactome studies mapping gene regulatory domains have clearly demonstrated the association of widely expressed genes with multiple cell type–specific enhancers,22 and endothelial subtype–specific enhancers have been reported for other pan-vascular transcribed genes.23

Flk1in10 Enhancer Contains Functional Binding Motifs for Ets, Gata, Rbpj, SoxF, and FoxC Transcription Factors
The mouse Flk1in10 enhancer shares significant sequence conservation between mammals and birds, but no detectable
conservation to reptiles or fish (Figure IA and IB in the online-only Data Supplement). We used ClustalW analysis to analyze the conserved enhancer sequences and identified conserved putative binding motifs for Ets, Gata, Rbpj, Sox, and Fox, which were then functionally verified by electrophoretic mobility shift assays (EMSA) using members of each transcription factor family known to be expressed in endothelial cells (Figure 3A–3D). Additional, less conserved motifs for Ets (ETS-a, c, e, i, and j), Gata (GATA-a and d), and Rbpj (RBPJ-b) were also tested in EMSA to ensure that functional yet poorly conserved binding sites were not overlooked. Five putative ETS motifs directly bound the Ets factor Ets2 (ETS-f, g, h, k, and l; Figure 3B), and 3 of 4 GATA motifs within the conserved enhancer directly bound Gata2 (GATA-b, c, and d; Figure 3C). All 3 putative SOX motifs directly bound Sox7 (SOX-a, b, and c), and both putative RBPJ motifs directly bound the protein (Figure 3C and 3D). The ETS-h motif was directly adjacent to a putative Forkhead binding site (FOX-b), forming a FOX:ETS motif (Figure 3). However, although a composite oligo containing both FOX-a and b motifs bound Foxc2, mutations to the FOX-b site did not ablate this binding (Figure 3D), suggesting that only the distal FOX-a site was required for Foxc2 binding and that the putative FOX:ETS motif may not be functional.

\textbf{FLK1in10 Enhancer Activity Requires Both ETS and GATA Transcription Factors}

Several Ets transcription factors play crucial roles in vascular specification, and all described endothelial cell enhancers contain multiple ETS-binding motifs. To confirm that Ets binding was required for FLK1in10 activity, all 5 functional ETS motifs were mutated (sequence information is available in the Materials and Methods in the online-only Data Supplement; EMSA was used to validate all mutations), and activity of the modified mouse enhancer (mutETS-f, g, h, k, and l) was tested in transgenic zebrafish (Figure 4A). As expected, the ablation of the functional ETS motifs resulted in a total loss of enhancer activity in the vasculature, supporting the essential role of Ets factors in vascular gene activation.

Both the FLK1 intron1 and dorsal multipotent mesodermal enhancers also contain conserved GATA motifs, and enforced Gata2 expression can induce FLK1 expression in hemangioblast culture conditions. To study the potential role for Gata transcription factors in the direct activation of the FLK1in10 enhancer, we mutated all GATA-binding motifs within the enhancer and tested the activity of the modified enhancer (mutGATA-all) in transgenic mouse and zebrafish (Figure 4A). Mutation of just the 2 conserved motifs, GATA-b and GATA-c (mutGATA-b,c), resulted in similar patterns of expression, although a low percentage of transgenic fish had detectable enhancer activity in intersegmental vessels (Figure 4A). To confirm these observations, we generated 11 mouse embryos transgenic for the FLK1in10mutGATA-b,c construct (Figure 4B–4F). All transgenic embryos showed detectable enhancer activity, but vascular expression was nearly entirely ablated in these embryos, limited to weak expression in a small number of vessels within the head region of 2 embryos (Figure 4C and 4D). In the remaining 9 transgenic embryos, enhancer activity was completely restricted to nonvascular tissues (Figure 4E and 4F).
Arterial Restriction of Flk1in10 Enhancer Activity Is Achieved by RBPJ-Mediated Repression in Veins
Ets and Gata transcription factors are expressed throughout the vasculature and regulate many enhancers active in both arterial and venous endothelial cells. Consequently, we hypothesized that additional binding motifs within Flk1in10 may be responsible for the arterial restriction of enhancer activity. We have previously demonstrated that activity of the arterial-specific Flk1in10 enhancer requires for vascular-specific activity of the Flk1in10 enhancer.

We next examined the consequences of morpholino knockdown of the zebrafish gene gata2a on the expression of the tg(Flk1in10:GFP) fish line (Figure 4G and 4H). Gata2a homozygous mutant zebrafish display defective trunk circulation and aorta morphogenesis defects, suggesting a role for Gata2 in arterial differentiation, although arterial and venous markers were unaffected. Knockdown of gata2a in tg(Flk1in10:GFP) transgenic zebrafish resulted in reduction (at low morpholino concentration) and ablation (at high morpholino concentration) of reporter gene expression (Figure 4G). Similar to gata2a mutant fish, arterial and venous differentiation occurred normally after morpholino-induced gata2a knockdown, and arterial–venous identity was maintained (Figure VI in the online-only Data Supplement). Because kdr, not kdrl, is the evolutionarily conserved ortholog of mouse Flk1, we also investigated whether gata2a deletion altered kdr expression. Similar to previous reports for kdrl (the functional equivalent of Flk1 in fish), expression of kdr was notably reduced, but not ablated, after gata2a deletion (Figure 4H). Conversely, knockdown of the related gata1a gene did not affect reporter gene expression (Figure VI in the online-only Data Supplement). These results support a requirement for Gata transcription factor binding for vascular-specific activity of the Flk1in10 enhancer.

Figure 2. The mouse Flk1in10 enhancer directs arterial-restricted expression in transgenic zebrafish. A, Schematic representation of the Flk1in10:GFP transgene. B, The mouse Flk1in10:GFP transgene directs endothelial cell–specific expression in transgenic zebrafish line tg(Flk1in10:GFP) at 24 h post fertilization (hpf). C–E, Tg(Flk1in10:GFP;kdr:HRAS-mCherry) zebrafish expresses GFP in most endothelial cells at 30 hpf (C). At 48 hpf, GFP expression in the axial vessels becomes restricted to the dorsal aorta and to a subset of intersegmental vessels, corresponding to the developing intersegmental arteries (ISA; D; see also Movie I in the online-only Data Supplement). At 72 hpf, little dorsal aorta expression could be detected, while GFP expression is maintained in the ISA and intersegmental veins (ISVe) (E). F, Bar chart detailing GFP expression pattern in the ISA and intersegmental veins (ISVe) at 72 hpf. Represents a total of 18 embryos, ISA and ISVe identity established by using kdr:HRAS-mCherry expression to determine whether each vessel connected to dorsal aorta or posterior cardinal vein. Any detectable level of GFP expression constituted positive, and Figure III in the online-only Data Supplement details analysis methods. bc indicates blood cells; da, dorsal aorta; dlav, dorsal longitudinal anastomotic vessel; dv, dorsal vein; GFP, green fluorescent protein; isv, intersegmental vessels; ISVe, intersegmental vein; and vv, ventral vein.

Arterial restriction of Flk1in10 enhancer activity is achieved by RBPJ-mediated repression in veins. Ets and Gata transcription factors are expressed throughout the vasculature and regulate many enhancers active in both arterial and venous endothelial cells. Consequently, we hypothesized that additional binding motifs within Flk1in10 may be responsible for the arterial restriction of enhancer activity. We have previously demonstrated that activity of the arterial-specific Dil4 enhancer, which is regulated by Notch intracellular domain (NICD) and Sox factors, requires functional binding motifs for vascular-specific activity of the Flk1in10 enhancer. Consequently, we hypothesized that additional binding motifs within Flk1in10 may be responsible for the arterial restriction of enhancer activity. We have previously demonstrated that activity of the arterial-specific Dil4 enhancer, which is regulated by Notch intracellular domain (NICD) and Sox factors, requires functional binding motifs for both Rbpj and Sox transcription factors. Therefore, we investigated the role of the RBPJ- and SOXF-binding motifs in the Flk1in10 enhancer. Surprisingly, unlike for the Dil4 enhancer, mutations to all RBPJ and SOX motifs (mutRBPJ;mutSox) did not result in the ablation of transgene expression in transgenic zebrafish and mice (Figure 5A–5D). However, in contrast to E12 mice transgenic for the Flk1in10 wild-type (Flk1in10-WT) enhancer, in which enhancer activity was restricted to arterial endothelial cells, the Flk1in10mutRBPJ;mutSox enhancer directed reporter gene expression to both arterial and venous endothelial cells. These results suggest that either the RBPJ or SOX motifs bind factors that repress the activity of the Flk1in10 enhancer in venous endothelial cells.

Because SoxF transcription factors are primarily arterial in mammals, we tested a second construct in which only the RBPJ sites were mutated (mutRBPJ). Again, expansion of enhancer activity into venous endothelial cells was seen in the majority of transgenic mice, suggesting that venous repression of Flk1in10 was mediated by Rbpj binding (Figure 5E). Of the 2 functional RBPJ motifs in the Flk1in10 enhancer, RBPJ-b bound most robustly in EMSA (Figure 3C). However, this motif was not well conserved in the Flk1in10 chicken.
Figure 3. The Flk1in10 enhancer contains cis-motifs that bind Etv2, Gata2, Rbpj, Sox7, and Foxc2 transcription factors. 

**A**, Multispecies alignment of the conserved region of the Flk1in10 enhancer. Colored sequences depict confirmed consensus binding motifs, and gray sequences depict motifs that did not bind in electrophoretic mobility shift assay.

**B**, Radiolabeled oligonucleotide probes encompassing Flk1in10 ETS-f (lanes 1–4), ETS-g (lanes 5–8), ETS-h (lanes 9–12), ETS-k (lanes 13–16), and ETS-l motifs (lanes 17–20) were bound to recombinant Etv2 proteins. All proteins efficiently bound to labeled probes (lanes 2, 6, 10, 14, and 18) were competed by excess unlabeled self-probe (wt, lanes 3, 7, 11, 15, and 19) but not by mutant self-probe (mu, lanes 4, 8, 12, 16, and 20).

**C**, Radiolabeled oligonucleotide probes encompassing Flk1in10 GATA-b (lanes 1–4), GATA-c (lanes 5–8), GATA-d (lanes 9–12), RBPJ-a (lanes 13–16), and RBPJ-b motifs (lanes 17–20) were bound to recombinant Gata2 and Rbpj proteins. All proteins efficiently bound to labeled probes (lanes 2, 6, 10, and 14) were competed by excess unlabeled self-probe (wt, lanes 3, 7, 11, and 15) but not by mutant self-probe (mu, lanes 4, 8, 12, and 20).

**D**, Radiolabeled oligonucleotide probes encompassing Flk1in10 SOX-a (lanes 1–4), SOX-b (lanes 5–8), SOX-c (lanes 9–12), and FOX-a/b (lanes 13–19) were bound to recombinant Sox7 and Foxc2 proteins. All proteins efficiently bound to labeled probes (lanes 2, 6, 10, 14, and 18) were competed by excess unlabeled self-probe (wt, lanes 3, 7, 11, 15, and 19) but not by mutant self-probe (mu, lanes 4, 8, 12, 16, and 20). For FOX-a/b, mutant self-probe with mutations to both FOX motifs (mu A+B) or to FOX-a could not compete with labeled probes (17, 19), whereas a mutant self-probe with mutations to FOX-b (mu B) was still able to robustly compete with labeled probe (18).
enhancer sequence, and the orthologous chicken RBPJ-b motif could not directly bind RBPJ in EMSA (Figure VIIA in the online-only Data Supplement). Therefore, we also tested the activity of the orthologous chicken Flk1in10 enhancer. Similar to mouse Flk1in10mutRBPJ, the chicken enhancer was robustly active in both arterial and venous endothelial cells (Figure 5F; Figure VIIB in the online-only Data Supplement). These results indicate that functional RBPJ-binding motifs are required for the repression of Flk1in10 enhancer activity in venous endothelial cells. Although Rbpj forms an activation complex with NICD, it can also inhibit transcription through the binding of corepressors and recruitment of repression complexes in the absence of NICD.31 Because the expression of Notch receptors Notch1 and Notch4 is greatly enriched in the arterial endothelium, our results suggest that the low levels of Notch signaling in venous cells result in the formation of repressive complexes at the RBPJ motifs of the Flk1in10 enhancer. Supporting this, chromatin immunoprecipitation...
Figure 5. Rbpj is required for arterial restriction of the Flk1 enhancer. A and B, Summary of reporter gene expression detected in (A) 48 h post fertilization (hpf) Tol2-mediated mosaic transient transgenic zebrafish embryos and (B) embryonic day (E) 12 transient transgenic mice. C–F, Whole-mount (top) and transverse sections (bottom) of representative E12 X-gal–stained transient transgenic embryos expressing mouse Flk1 WT (C), mouse Flk110mRBPJ/SOX (D), mouse Flk110mRBPJ (E), and chicken Flk110 WT (F). Lines marked i and ii on C mark the approximate location of transverse sections in C–F. A indicates artery; cv, cardinal vein; da, dorsal aorta; and v, veins. G–I, Analysis of the effects of scrambled (G), 1.25 ng rbpj morpholino (MO) oligonucleotides (H), and Notch signaling inhibitor N-[N-3,5-difluorophenacetyl]-L-alanyl-S-phenylglycine methyl ester (DAPM) (I) in 72 h post fertilization (hpf) tg(Flk110:GFP) embryos. MO-mediated knockdown of rbpj results in expansion of Flk110:GFP expression into the caudal vein, the posterior cardinal vein, and intersegmental vein. da indicates dorsal aorta; GFP, green fluorescent protein; ISA, intersegmental artery; ISVe, intersegmental vein; and pcv, posterior cardinal vein.
may merely represent the acquisition of repression, not the suggested a possible positive role for Notch, yet these results expression after DAPM-mediated sequestration of NICD and fish may inadvertently mask the minor effects of acces-sory transcriptional activators. Furthermore, loss of Rbpj in zebrafish resulted in an expansion of Flk1in10:GFP expression at 72 hours post fertilization (Figure 5I), providing further evidence that Rbpj is able to repress Flk1in10 expression in the absence of Notch signaling. A schematic model summarizing these results is found in Figure VIII in the online-only Data Supplement.

Potential Roles for Sox and Notch in Promoting Activity of Flk1in10 in Arterial Cells

Although our results suggest that only Ets and Gata factors transcriptionally activate Flk1in10 expression in endothelial cells, this may not preclude the involvement of other positive regulatory factors. All 3 characterized Flk1 enhancer elements contain essential ETS- and GATA-binding motifs, yet the activity of these enhancers overlaps only during the very early stages of vascular development. Because intact Notch signaling, essential for arterial differentiation, is itself downstream of VEGF through the Flk1 receptor, and loss of Notch signaling resulted in ablation of Flk1in10:GFP expression at 72 hours post fertilization (Figure 5I), we wanted to investigate whether Rbpj/NICD binding could also provide a positive input to the Flk1in10 enhancer. The potential for such a role is supported by recent analysis of other arterial-restricted enhancers that have demonstrated activating roles for Rbpj/Notch and Sox regulatory pathways, demonstrating a direct link between the Notch and VEGF signaling pathways, and a novel regulatory role for Rbpj in repression of arterial identity in venous endothelial cells.

We have previously described an enhancer for the Notch ligand Dll4 (Dll4in3) which is also active in arterial endothelial cells. Although both enhancers can be described as directing arterial expression within the vasculature, they do not direct identical expression patterns: Dll4in3 is arterial specific throughout development, whereas Flk1in10 starts pan-vascular then gradually becomes arterially restricted. It is therefore unsurprising that the transcriptional mechanisms regulating these 2 enhancers are divergent. Rbpj plays no clear repressive role in Dll4in3 regulation, although Notch-dependent Rbpj activation in combination with SoxF factors is essential for enhancer activity. Conversely, our study demonstrates a role for Rbpj in repression of Flk1 expression in veins, in line with the view of Rbpj as a mediator of default repression in the absence of Notch signaling. These differences of partial depletion of GATA in conjunction with RBPJ and SOX on Flk1in10 activity in transgenic zebrafish and mice. First, we generated a Flk1in10 construct in which one conserved GATA site (GATA-c) was left functional, whereas the other sites were mutated (mutGATAa,b,d). In both transgenic zebrafish and mice, the presence of a single functional GATA-c site resulted in increased enhancer activity in endothelial cells compared with mutGATA-all and mutGATA-b,c (Figure 6A–6E compared with Figure 4A and 4B). However, the expression levels were reduced compared with the WT Flk1in10 enhancer (Figure 6A and 6B). We further mutated this construct to also ablate the binding sites for SOX and RBPJ, creating Flk1in10mutGATA-a,b,d/ RBPJ/SOX. Surprisingly, the additional mutation of the SOX and RBPJ sites resulted in a complete loss of reporter gene expression in both zebrafish and mouse transgenic embryos (Figure 6). These results suggest that, when the absolute binding levels of Gata are reduced, Rbpj/NICD and SoxF factors may play a transcriptionally activating role. Further transgenic analysis, this time using Flk1in10 constructs containing alternative mutations ablating either the GATA-b motif alone or in combination with SOX and RBPJ, resulted in similar patterns of transgene expression in both zebrafish and mouse models (Figure 6). These results therefore suggest that the RBPJ- and SOX-binding motifs, although not sufficient for enhancer activity alone, may provide the Flk1in10 enhancer with some level of positive regulatory input in arterial endothelial cells, alongside the more important role of Rbpj in the suppression of Flk1in10 in the vein.

Discussion

In this study, we have identified a novel endothelial cell enhancer within the 10th intron of Flk1. Similar to other Flk1 regulatory elements, this enhancer requires both Ets- and Gata-binding motifs for transcriptional activity. However, Flk1in10 enhancer activity was restricted to the arterial compartment through both repressive and activating signals downstream of Rbpj/Notch and Sox regulatory pathways, demonstrating a direct link between the Notch and VEGF signaling pathways, and a novel regulatory role for Rbpj in repression of arterial identity in venous endothelial cells.
in expression outcome likely reflect the distinct upstream signals regulating the Notch and VEGF pathways in vascular development and further support the idea that neither the venous nor the arterial endothelial cell fully represents a default vascular state onto which the alternative identity is actively acquired.

Although Ets factors have long been known to be crucial for endothelial cell specification, recent studies have also implicated the Ets factor Erg in arterial specification downstream of VEGF. It remains possible that Erg may contribute to the arterial-restricted activity of GATA sites in Flk110.
of Flk1in10. However, the ETS-binding motifs were present and functional in the Flk1in10mutRBPJ/mutSOX enhancer, which was active in both arterial and venous endothelial cells, and the Flk1in10mutGATA/mutRBPJ/mutSOX enhancer, which was not active in endothelial cells at all. Consequently, although we cannot absolutely rule out an indirect role for Erg, it is clear that ETS motifs are not sufficient to achieve arterial specification or enhancer activation in the absence of other transcription factors.

Ablation of Gata2 specifically in endothelial cells in both mice and zebrafish does not recapitulate the Flk1 null phenotype, although later vascular differentiation was impaired.\(^8\)\(^9\)\(^15\) Although it is theoretically possible that the Flk1 locus could be independent of Gata factors via additional, undiscovered regulatory elements, this seems unlikely: all 3 known Flk1 enhancers require GATA-binding motifs to function, and expression of the orthologous kdr and kdrl genes was diminished in zebralsh after disruption of gata2a. Notably, Gata2 is not the only member of the Gata family expressed in the vasculature: both Gata3 and Gata6 are also expressed in endothelial cells during vascular development.\(^3\) Therefore, it is likely that additional members of the Gata transcription factor family can contribute to Flk1 regulation and at least partially compensate in the absence of Gata2.

Although it is known that VEGF and Notch both act in a conserved genetic pathway to promote arterial differentiation, and VEGF-mediated induction of Notch receptors requires intact Notch signaling, some models have suggested that Notch signaling can actively repress Flk1 levels. This was first proposed in studies of the angiogenic front in the postnatal retina, where decreased Notch levels in tip cells correspond with increased angiogenic sprouting and filopodia projections.\(^2\) However, the extent of direct Notch inhibition of Flk1 varies significantly in different experimental contexts, and although Flt4 (Vegfr3) expression inversely correlates with areas active in Notch signaling, the same is not true for Flk1.\(^6\)\(^9\) It is therefore likely that any repression of Flk1 expression downstream of active Notch during angiogenesis may reflect indirect repression via Flt4 instead of active Notch-mediated repression of Flk1 expression.\(^6\)\(^9\)\(^15\)

Although enhancer studies primarily focus on the transcription factors essential for activity, it is likely that much of the crucial information governing vascular differentiation, patterning, and tissue specificity comes from nonessential, and consequently harder to assay, regulatory inputs. This research demonstrates that enhancer regulation of Flk1 is more nuanced, and more complicated, than a simple on/off transcription factor switch, reflecting the reality of expression of an essential and multifaceted receptor within the heterogenous vasculature. Multiple enhancers, binding multiple transcription factors, permit different layers of transcriptional regulation and allow many different upstream cues to subtly alter expression levels. The arrangement of binding motifs within the Flk1in10 enhancer may enable Notch signaling to subtly suppress Flk1 expression in venous cells, whereas amplifying it in arterial cells, therefore providing a way to ensure correct arterial–venous differentiation without detrimentally influencing the other essential functions of the VEGF pathway in the vasculature.

Acknowledgments
We thank N. Ahituv for providing Gateway-compatible vectors and M. Shipman for help with imaging.

Sources of Funding
This work was supported by Ludwig Institute for Cancer Research Ltd and the Medical Research Council (MR/J007765/1) (N. Sacilotto, K. Liu, G. Bou-Gharios, and S. De Val) and the Wellcome Trust (090552/z/09/z) (C. Preece and B. Davies).

Disclosures
None.

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**Highlights**

- Novel endothelial enhancer for Flk1 located in the 10th intron.
- Flk1in10 enhancer expression becomes restricted to arterial endothelial cells during development.
- Notch pathway transcriptional effector RBPJ represses Flk1 enhancer expression in venous endothelial cells.
An Intronic Flk1 Enhancer Directs Arterial-Specific Expression via RBPJ-Mediated Venous Repression
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Arterioscler Thromb Vasc Biol. 2016;36:1209-1219; originally published online April 14, 2016; doi: 10.1161/ATVBAHA.116.307517
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplemental Figure I.
A. Schematic representation of the human KDR locus from UCSC ENCODE Browser. This information was used to identify the intron 10 region as a putative enhancer. HUVEC specific H3K4Me1 peaks in light blue, HUVEC specific DNase I HS peaks in red, sequence conservation between human and mouse, opossum, chicken and zebrafish indicated by vertical black lines. Region homologous to mouse Flk1in10 enhancer indicated with red bar, regions homologous to mouse Flk1 intron 1 enhancer and DMME enhancers marked with black horizontal bars.
B. Schematic representation of the mouse Flk1 locus from UCSC ENCODE Browser. E14.5 heart specific H3K4Me1 upper peaks in maroon, heart specific DNase I HS lower peaks in maroon, sequence conservation between mouse and human, opossum, chicken and zebrafish indicated by vertical black lines. Region encompassing the Flk1in10 enhancer indicated with red bar, regions encompassing Flk1 intron 1 enhancer and DMME enhancers marked with black horizontal bars. Note that other untested intronic regions also have enhancer-associated marks indicative of putative enhancer elements. Genome browser screen shots from http://genome.ucsc.edu.
Supplemental Figure II.

A. Transverse sections through different levels of the heart of E9, E10 and E12 Flk1in10-LacZ transgenic mouse embryos. * indicates endocardium, arrowhead indicates myocardium, ac common atrial canal, avc atrial-ventricular canal, ec endocardial cushion, la left atria, lv left ventricle, ra right atria, rv right ventricle, ot outflow tract, tw trabecular wall.

B. Time-course of organs from Flk1in10-LacZ transgenic mice embryos, P6 pups and 10 week old adult mice. All organs were dissected and stained separately for X-gal, with exception of P6 retina (isolectin) picture, in which the P6 retina depicted to the right was stained for expression of the pan-endothelial marker isolectin b4 conjugated to DyLight 594 (red).
Supplemental Figure III.

A. Detailed example of the analysis of arterial-venous identity of intersegmental sprouts used in Figure 2. Pictures denote single image of a representative 72 hpf Tg(Flk1in10:GFP;kdrl:HRAS-mCherry) embryo. Top panel denotes mCherry image only, in which the pan-vascular kdrl:HRAS-mCherry transgene is expressed in all intersegmental vessels as well as the dorsal aorta (DA) and posterior cardinal vein (PCV). Using this image we were able to assess the identity of each intersegmental vessel by whether it connects to the DA (arterial) or PCV (venous). The transparency of the embryo meant that we image two intersegmental vessels for each segment, one in the foreground denoted by UPPERCASE and the other in the background, denoted by bracketed lowercase. The middle image denotes both GFP and mCherry expression, and the bottom image just GFP expression. For each intersegmental vessel detected by kdrl:HRAS-mCherry expression, we scored whether GFP expression was detected (Y) or not (N). Vessels in foreground recorded as UPPERCASE, vessels in background as bracketed lowercase. * represents GFP in one cell, still recorded as GFP expression (Y) by our method.

B. Creation of identity and GFP score used to create the bar chart in Figure 2. Records only foreground intersegmental identity and GFP expression.

Identity score: A V V A A V V A A A
GFP score: Y Y N Y Y N N Y Y Y Y
Supplemental Figure IV.

Loss of circulation does not prevent arterial restriction of Flk1in10:GFP expression. Analysis of the effect of 4ng tnnt2 MO in 28, 48 and 72 hpf tg(Flk1in10:GFP;kdrl:HRAS-mCherry) embryos. kdrl:HRAS-mCherry marks all vessels. ISA intersegmental artery, ISVe intersegmental vein.
Supplemental Figure V
Transverse sections through Flk1 intron1-LacZ transgenic embryo at E12 demonstrates transgene expression in both venous and arterial endothelial cells. A artery, ca carotid artery, jls jugular lymph sac, jv jugular vein, ta tail artery, tv tail vein, v vein.
Supplemental Figure VI.
A. Analysis of scrambled, 2.25ng and 3.375 ng gata2a MO in 26 hpf WT zebrafish embryos, using whole-mount in situ hybridization with probes against arterial marker dll4, venous marker flt4 and kdr1.
B. Analysis of 2.5ng gata1 MO in 36 hpf tg(Flk1in10:GFP) embryos.
Supplemental Figure VII.

**A.** Radiolabeled oligonucleotide probe encompassing mouse Flk1in10 RBPJ-a (lanes 1-4) was able to robustly bind to recombinant Rbpj protein (lane 2), but the orthologous chicken Flk1in10 RBPJ-a (lanes 5-8) and chicken Flk1in10 RBPJ-b (lanes 9-12) motifs were unable to bind in the same experimental conditions (lane 6 and 10). Rbpj protein was competed by excel unlabeled self-probe (lane 3) but not by mutant self-probe (4). Experiment was done in parallel, n=2.

**B.** Transverse sections from representative E12 X-gal stained transient transgenic embryos expressing mouse Flk1in10-WT, mouse Flk1in10mRBPJ/SOX, mouse Flk1in10mRBPJ and chicken Flk1in10 WT. Upper line shows carotid artery and jugular vein, lower line shows tail artery and paired veins. Ca carotid artery, jls jugular lymph sac, jv jugular vein, ta tail artery, tv tail vein.

**C.** Chromatin immunoprecipitation assays were performed with anti-Rbpj, anti-Notch1 and control immunoglobulin G (IgG) antibodies. The DNA content of the immunoprecipitates was analyzed by real-time PCR for Flk1in10. Results were normalized by input and IP enrichment expressed relative to the IgG. Error bars mean standard deviation of n=2.
Supplemental Figure VIII.
Schematic summarising the effects that perturbations to the Notch pathway have on the transcriptional activation of the Flk1in10 enhancer. NICD denotes Notch intracellular domain, MAM denotes Mastermind-like transcriptional co-activator, Co-R denotes co-repressors. Red angled arrow indicates transcriptional activation, black arrow with strike-through indicates transcriptional repression.
Cloning of Flk1in10 WT and mutant enhancers constructs

The 826 bp Flk1in10 enhancer was generated by PCR from mouse genomic DNA using Flk1in10 mouse F and R primers (primer sequence provide in table below). The orthologous 1012 bp chicken Flk1in10 enhancer was generated by PCR from chicken genomic DNA using the Flk1in10 chicken F and R primers (primer sequence provide in table below). PCR products were cloned into the pCR8 vector using the pCR8/GW/TOPO TA Cloning Kit (Invitrogen, K2500-20) following manufacturer’s instructions. The Flk1 intron 1 enhancer was generated by PCR from mouse genomic DNA using Flk1in1 mouse F and R primers and cloned in pCR8 as described.

Mutant versions of the mouse Flk1in10 enhancer were initially generated as custom-made, double-stranded linear DNA fragments (GeneArt® Strings™, Life Technologies) and were subsequently treated as PCR products described above. The sequences of each mutant enhancer DNA fragment are provided in the DNA fragment sequence information. Once cloning was confirmed, the enhancer sequence was transferred from the pCR8/GW/enhancer entry vector to a suitable destination vector using Gateway LR Clonase II Enzyme mix (Life Technologies, 11791-100) following manufacturer’s instructions. For mouse transgenesis, the enhancer was cloned into the hsp68-LacZ-Gateway vector (provided by N. Ahituv). For zebrafish transgenesis, the enhancer was cloned into the E1b-GFP-Tol2 vector (provided by N. Ahituv).

Generation and analysis of transgenic fish

F0 transient mosaic transgenic zebrafish embryos were generated by the Tol2 system (1). Briefly, 0.5 nl of 50 ng/µl Tol2 transposase capped mRNA (mMESSAGE mMACHINE® SP6 Kit, Ambion) and 60 ng/µl pE1b/enhancer/GFP expression vector were injected into 1-cell embryos obtained by natural spawning of wild type (WT) adult zebrafish raised and maintained at 28.5°C in system water. Embryos were maintained in E3 medium (5 mM NaCl; 0.17 mM KCl; 0.33 mM CaCl2; 0.33 mM MgSO4) at 28.5 °C.

The Flk1in10:GFP stable line tg(Flk1in10:GFP) was generated from an initial outcross of adult F0 carriers generated by the Tol2 system described above. GFP positive embryos from these initial outcrosses (F1 generation) were analyzed for expression, and two representative lines selected. The tg(Flk1in10:GFP) line was intercrossed with tg(kdrl:HRAS-mCherry)1 to enable visualization of the entire vasculature.

To image, all embryos were dechorionated and anesthetized with 0.1% tricaine mesylate. For analysis of transient transgenic zebrafish, single embryos were transferred into a flat bottom 96-well plate plate, and GFP reporter gene expression screened with a Zeiss LSM 710 confocal microscope at 46-50 hpf. The total number of injected fish, the total number of fish with any detectable GFP expression, and the total number of fish with any detectable GFP expression in the vasculature were all noted. Whole fish were imaged using the “tile scan” command, combined with Z-stack collection under a confocal microscope Zeiss LSM 710 MP (Carl Zeiss) at 488nm excitation and 509nm emission (EGFP) and 587 nm and 610 nm (mCherry), respectively. Movies were generated by collecting images at 24FPS under a confocal microscope Zeiss LSM 710 MP (Carl Zeiss) using the “time series” command.

To assess GFP expression patterns in 72 hpf tg(Flk1in10:GFP; kdrl:HRAS-mCherry) zebrafish embryos, still images were taken and assessed. The identity of
the intersegmental vessel was established using the expression of the pan-vascular kdr:HRAS-mCherry reporter and identifying whether the vessel ran into the dorsal aorta (intersegmental artery) or cardinal vein (intersegmental vein). Each vessel was then assessed for GFP expression.

**Generation and analysis of transgenic mice**

All animal procedures were approved by local ethical review and licensed by the UK Home Office. Transgenic mice were generated by oocyte microinjection as described previously.

Transgenic mouse embryos were collected along with yolk sac and placenta. After dissection, samples were rinsed in ice-cold 1× PBS and fixed in 2% PFA, 0.2% glutaraldehyde, 1× PBS at 4°C as indicated below:

| Age       | Fixation time | Further processing                                      |
|-----------|---------------|--------------------------------------------------------|
| E7.5-8.5  | 10 min        | Embryos were rinsed twice in 1× PBS, then incubated in PBS for 30 min at 4 °C |
| E8.5-9.0 / hindbrain | 20 min |                                            |
| E9.5      | 30 min        |                                                        |
| E10-11.5  | 60 min        |                                                        |
| E12.5     | 1.5 h         | Embryos were rinsed twice in PBS, than incubated 2 x 30 min in rinse solution (0.1% sodium deoxycholate, 0.2% Nonidet P-40, 2 mM MgCl₂, 1× PBS) at 4 °C |
| E13.5 / organs | 2 h | sodium deoxycholate, 0.2% Nonidet P-40, 2 mM MgCl₂ and 1× PBS. After staining, embryos were rinsed through a series of 1× PBS washes, then fixed overnight in 4% paraformaldehyde at 4 °C. |
| E14.5     | 2.5 h         |                                                        |
| E15.5     | 3 h           |                                                        |
| E16.5-17.5| 4 h           |                                                        |

Retinas were fixed in 4% PFA for 1h on ice.

After fixation and rinse, embryos were stained overnight at room temperature in 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside solution (X-gal) containing 5 mM potassium ferrocyanide, 5 mM ferricyanide, 0.1% sodium deoxycholate, 0.2% Nonidet P-40, 2 mM MgCl₂ and 1× PBS. After staining, embryos were rinsed through a series of 1× PBS washes, then fixed overnight in 4% paraformaldehyde at 4 °C.

Imaging of whole embryos and organs was performed using a stereo microscope (Leica M165C) equipped with a ProGres CF Scan camera (Jenoptik) and ProgRes CapturePro software (Jenoptik). For each enhancer, embryos were also sectioned for histological analysis to investigate X-gal staining patterns. For histological analysis, embryos were dehydrated through a series of ethanol washes, cleared by xylene and paraffin wax-embedded. 5 or 6-μm sections were prepared, de-waxed, and counterstained with nuclear fast red (Electron Microscopy Sciences). Co-staining for Endomucin and β-galactosidase was performed on de-waxed sections using a dilution of 1:100 rat anti-Endomucin antibody (Santa Cruz, sc-65495) and 1:100 mouse anti-β-galactosidase (MP Biomedicals, 559761) in PBS as described previously.

Retinas and hindbrains were incubated in blocking buffer (0.5% Tween + 1% Normal Horse Serum in PBS) for 1h and stained with DyLight 594 GSL I-B4 Lectin (Vector, DL-1207) in PBS+ 0.5% Tween for 2h followed by 5 x 10 min washes in PBS + 0.5% Tween. Retinas and hindbrains were then flat mounted using VECTASHIELD mounting medium (Vector, H-1200) and imaged using using the “tile scan” command, combined with Z-stack collection under a confocal...
microscope Zeiss LSM 710 MP (Carl Zeiss) at 590nm excitation and 617nm emission.

The placenta was used for genotyping. Tissue samples were incubated overnight at 55°C with 500 µL GNT buffer (50mmol/L KCl, 1.5mmol/L MgCl₂, 10mmol/L Tris-pH8, 0.01% gelatin, 0.45% nonidet P40, 0.45% Tween) and proteinase K (10mg/ml). Afterwards, the solution was heated to 95°C for 30 minutes and centrifuged for 1 minute at maximum rpm in a benchtop centrifuge. 0.5 µL of this supernatant were subsequently used in a PCR reaction with the GoTag Green master mix (Promega, M7122) using LacZ PCR primer F and R (sequence provide in table below).

**Morpholinos and Chemical Treatments.**

Antisense morpholino oligonucleotides were as described previously (Gata1 ATG MO and gata2a splice MO⁴; Sox7 ATG MO and SOX18 ATG MO⁵; RbpJ ORF MO ¹,²,⁶, tnnt2 MO²,⁷). MOs were injected into 1- to 2-cell wild-type or tg(Flk1in10:GFP) embryos, at a concentration depicted in the figure legends (maximum injection volume of 2 nl). For inhibition of Notch signaling embryos were incubated with 100 µM DAPM (Calbiochem) starting at 26 hpf.

**Zebradfish whole mount in situ**

In situ hybridization procedures used the following probes: flt⁴,⁵,⁸, dll⁴,⁵,⁹, kdr¹⁰. kdr probe was generated as a DNA fragment (sequence listed below) from GeneArt® Strings™ (Life Technologies), cloned using the pCR2/TOPO/TA cloning kit (Invitrogen 450641) and transcribed using SP6 and T7. In situ analysis was conducted as previously described¹¹. Briefly, embryos were collected at 26hpf and fixed overnight at 4 °C in 4% paraformaldehyde. Fixed embryos were dehydrated and stored at -20 °C in 100% ethanol. When needed embryos were rehydrated in PBST, treated with 10µg/ml proteinase K (Sigma) for 10 minutes, followed by two PBST washes. Thereafter, the embryos were fixed with 4% PFA for 20 min and washed five times with PBST. Embryos were transferred into hybridization solution (50% formamide, 5 × SSC, 0.1% Tween 20, 50 µg/ml heparin, 500 µg/ml of tRNA adjusted, 10 mM citric acid) for 2 hours at 65 °C, then transferred into diluted antisense riboprobe/hybridization solution and incubated overnight at 65 °C. Probes were removed and embryos transferred to a Biolane HT-1 in situ machine (Intavis). Embryos were washed through a dilution series into 2 x SSC followed by 0.2 x SSC at 65 °C. The embryos were then taken through room temperature dilution washes into 100 % MABT (0.1M Maleic Acid, 0.15 M NaCl, pH 7.5). Non-specific sites were blocked with MAB block (MABT with 2% Boehringer block reagent) at room temperature and incubated for 15 hours with anti-DIG antibody (Roche) at 1:2000 at 4 °C, before washing in MABT. Prior to staining, embryos were washed in AP buffer and the in situ signal developed at room temperature with BM Purple (Roche). Staining was stopped as appropriate by fixation in 4% paraformaldehyde. Embryos were transferred to 80% glycerol for imaging and storage.

**Electrophoretic mobility shift assay**

Electrophoretic mobility shift assay (EMSAs) were performed as described previously ³. Proteins were made using the TNT Quick Coupled Transcription/Translation system as described in the manufacturer’s directions. The full length Sox7 and Gata2 were in the pCITE2 plasmid, and transcribed using T7 polymerase. Etv2 was in the pCS2 plasmid, and transcribed using Sp6 polymerase.
Foxc2 was in the pCR2.1 plasmid and transcribed using T7, Rbpj was in the pcDNA3.1 plasmid and transcribed using T7.

To label the probe, double stranded oligonucleotides were labeled with 32P-dCTP, using Klenow (Promega) to fill in overhanging 5’ ends, and purified on a non-denaturing polyacrylamide-TBE gel. 20 µl binding reactions consisted of 3-5 µl protein or lysate control and 2 µl 10X binding buffer (40mM KCl, 15 mM HEPES pH 7.9, 1 mM EDTA, 0.5 mM DTT, 5% glycerol). For Etv2 and Rbpj, 0.5 µg of poly dl-dC was used. For Foxc2, 0.25µg of poly-dI-dC was used, and for Sox7, 0.25µg poly-dG-dC was used. For competitor lanes, a 100-fold excess of competitor DNA was added in a volume of 1µl. Binding reactions were incubated at room temperature for 20 minutes before the addition of radiolabeled probe, after which they were incubated an additional 20-40 minutes. Gels were electrophoresed on a 6% non-denaturing polyacrylamide gel.

Chromatin immuno precipitation (ChIP)

Mouse aortic and venous endothelial cells (C57BL/6 mouse primary vein endothelial cells C57-6009 and mouse primary aortic endothelial cells C57-6052, CellBiologics) were grown to 70% confluency in Endothelial Cell Medium (CellBiologics M1168), and fixed in 1% methanol-free PFA at 4C for 10 min. Cross-linked cells were washed twice with ice-cold PBS, carefully lysed in cell-lysis buffer supplemented with protease inhibitors (Sigma) to obtain intact nuclei. Nuclei were subsequently sedimented at 1000 x g for 5 min at 4C and lysed in nuclei lysis buffer (50mM Tris pH8.1, 10mM EDTA, 1% SDS). Chromatin was sonicated to obtain fragments of an average size of 400 bp using a Covaris sonicator. Diluted and pre-cleared chromatin was then immunoprecipitated with RbpJ, Notch1 and IgG antibodies. The ChIP was analysed by qPCR using primers described below in table. Results were normalized by input and IP enrichment expressed relative to the IgG. Error bars mean standard deviation of n=2.

PCR, EMSA and Morpholino primers

Lowercase indicate non-homologous 5’ tag to permit radioisotope labeling. Lowercase italics indicate mutated nucleotides.

| Oligo name       | Oligo sequence                                      |
|------------------|-----------------------------------------------------|
| LacZ PCR primer F | 5’-GTTGCAGTGCACGGCAGATACACTTGCTGA-3’                |
| LacZ PCR primer R | 5’-GCCACTGGTGTGGCCATAATTCAATTCGC-3’                |
| Flklin10 mouse F  | 5’-GCATGTCAGGATTTGACTTC-3’                         |
| Flklin10 mouse R  | 5’-CAGGATCGGCAATGGAAACAG-3’                        |
| Flklin10 chicken F | 5’-GCTGATCAAACAGTACATG-3’                          |
| Flklin10 chicken R | 5’-AGCACATATGCGATAGGGAG-3’                         |
| Flklin10 GATA-a F  | ctagGTCCTGAGGATACAGGGG                              |
| Flklin10 GATA-a mut F | ctagGTCCTGAGggACAGGGGG                            |
| Flklin10 GATA-b F  | ctagTAGACCTTGATAACCTGGG                            |
| Flklin10 GATA-b mut F | ctagTAGACCTTGGaAGGCTTGGG                        |
| Flklin10 GATA-c F  | ctagGGGCTAGGTTATCAGCTGCC                           |
| Flklin10 GATA-c mut F | ctagGGGCTAGGGgACTGGCTC GC                      |
| Flklin10 GATA-d F  | ctagTCCACAGATAAGGAGGAC                           |
| Flk1in10 GATA-d mut_F | ctagTCCCACAGggAAGGAGGAGC |
|------------------------|---------------------------|
| Flk1in10 ETS-a F       | ctagAAGGTCTCTGAGGATACAGGAGGA |
| Flk1in10 ETS-a mut_F   | ctagAAGGTCTCTGAGCcTACAGGAGGA |
| Flk1in10 ETS-b F       | ctagATACAGGAGGGAAGCAGTATTCT |
| Flk1in10 ETS-b mut_F   | ctagATACAGGAGGccAGCAGTATTCT |
| Flk1in10 ETS-c F       | ctagTTCTAAATTTGGAATGAGCAACAGA |
| Flk1in10 ETS-c mut_F   | ctagTTCTAAATTTGccTACAGGAGGA |
| Flk1in10 ETS-d F       | ctagACTTATTTGCAAGGAGGAGTAAAG |
| Flk1in10 ETS-d mut_F   | ctagACTTATTTGCAAGccAGTAAAG |
| Flk1in10 ETS-e F       | ctagAGAAGGTAAAGGAAACTTGGG |
| Flk1in10 ETS-e mut_F   | ctagAGAAGGTAAAGGccAAGTGGG |
| Flk1in10 ETS-f F       | ctagTTTTTGTTTAGAACAGCATGG |
| Flk1in10 ETS-f mut_F   | ctagTTTTTGTCTCCAATGGGACAG |
| Flk1in10 ETS-g F       | ctagCAGCAGAGGAGGAAACTTGGG |
| Flk1in10 ETS-g mut_F   | ctagCAGCAGAGGccAGAAGACTTGGG |
| Flk1in10 ETS-h F       | ctagACAAACAGGAAGTGGACTGC |
| Flk1in10 ETS-h mut_F   | ctagACAAACAGGccAGTGGACTGC |
| Flk1in10 ETS-i F       | ctagACAGAAAGTGGAAAGCTTGGG |
| Flk1in10 ETS-i mut_F   | ctagACAGAAGGAGGcAGCAGTGGG |
| Flk1in10 ETS-j F       | ctagGGCTGTTGTTTTTCTAAGGACGT |
| Flk1in10 ETS-j mut_F   | ctagGGCTGTTGTTTggCCTAAGGACGT |
| Flk1in10 ETS-k F       | ctagTTTTTGTCTTTAACAGCATGG |
| Flk1in10 ETS-k mut_F   | ctagTTTTTGCTAAGGTAAGGAGTAAAG |
| Flk1in10 RBPJ-a F      | ctagAGCAGGAGGAGGAAACTTGGG |
| Flk1in10 RBPJ-a mut_F  | ctagAGCAGGAGGccAGAAGACTTGGG |
| Flk1in10 RBPJ-a,b F    | ctagATTTCAAAACAGCAACACAGGAAGTGGGAATG |
| Flk1in10 RBPJ-a,b mut  | ctagATTTCAAAACAGCAACACAGGAAGTGGGAATG |
| a,b F                  | ctagATTTCAAAACAGCAACACAGGAAGTGGGAATG |
| Flk1in10 SOX-a F       | ctagAGTCTCCATTCAAAATTTGGA |
| Flk1in10 SOX-a mut_F   | ctagAGTCTCCATggTAAATTTGGA |
| Flk1in10 SOX-a,b F     | ctagAGAACAATTCAAGCTCAGT |
| Flk1in10 SOX-b mut_F   | ctagAGAACAATGggAGCTCAGT |
| Flk1in10 SOX-c F       | ctagTCAGTATTGTggGAGGAAAGT |
| Flk1in10 SOX-c mut_F   | ctagTCAGTATTGTggGAGGAAAGT |
| Flk1in10 FOX-a,b F     | ctagATTTCAAAACAGCAACACAGGAAGTGGGAATG |
| Flk1in10 FOX-a,b mut  | ctagATTTCAAAACAGCAACACAGGAAGTGGGAATG |
| a,b F                  | ctagATTTCAAAACAGCAACACAGGAAGTGGGAATG |
| Flk1in10 FOX-a,b mut a | ctagATTTCAAAACAGCgCgCAACACAGGAAGTGGGAATG |
| Flk1in10 FOX-a,b mut b | ctagATTTCAAAACAGCgCgCAACACAGGAAGTGGGAATG |
| ChIP Flk1in10 RBPJ-a F | caacagtgcgaaggtcccttcga |
| ChIP Flk1in10 RBPJ-a R | agcttgaatgcttgccttgcttgatcc |
| ChIP Flk1in10 RBPJ-b F | taggttttatcaatcctgcccttgatcc |
| ChIP Flk1in10 RBPJ-b R | accacactgcctcctgcttgatcc |
| Morpholinos | Name              | MO sequence                  | Reference |
|-------------|-------------------|------------------------------|-----------|
|             | GATA1 ATG MO      | CTGCAAGTGTAGTATTGAAGATG      | 4         |
|             |                   | TC                            |           |
|             | GATA2a splice MO  | CATCTACTCAACCAGTCTCGCTTT    | 4         |
|             |                   | TG                            |           |
|             | RBPJ MO           | CAAACTTCCCCTGTCAACAACAGGC    | 6         |
|             |                   | GC                            |           |
|             | scrambled MO      | CCTCTTACCTCAGTTACAATTTA      | 12        |
|             |                   | TA                            |           |
|             | Sox 7 ATG MO      | ACGCACTTATCAGAGGCGCCTATG     | 5         |
|             |                   | TG                            |           |
|             | Sox18 ATG MO      | TATTCATCCAGCAAGACCAACAAGCG  | 5         |
|             |                   | GC                            |           |
|             | Tnnt2 MO          | CATGTTTGCTCTGTACCTCAGACG     | 7         |
|             |                   | CA                            |           |

Sequence of synthesized DNA fragments

Mouse Flk1in10 WT

TGCATGTCAGATTGGACTCTCTCTCTGTCAGGAGGTGCGGAAAGGTCAGCCTCTGGTTATC
TCAGTTCTCTAGTGATAACCCTCGACACACTCGAAGACTTTGCAGAATCTGGGCAATTAA
AATTAGATGCGATACCCATCGCAGACACTTCTCGACAGACATCTGGGGCAATTAA
ATTTAGGAACCATGACGAGAGGTGCTGGTGAGCTGAGATCGAAACTTGGATGCTGGGTGGCTGGTGCTG
TCCTAAGCTATGCTGACTTTGTTGACTATACCGCTAAGCTGCGACTTTGCAGACACTTCTGGGGCAATTAA
AATTAGATGCGATACCCATCGCAGACACTTCTCGACAGACATCTGGGGCAATTAA
ATTTAGGAACCATGACGAGAGGTGCTGGTGAGCTGAGATCGAAACTTGGATGCTGGGTGGCTGGTGCTG
TCCCTAAGCTATGCTGACTTTGTTGACTATACCGCTAAGCTGCGACTTTGCAGACACTTCTGGGGCAATTAA
AATTAGATGCGATACCCATCGCAGACACTTCTCGACAGACATCTGGGGCAATTAA
ATTTAGGAACCATGACGAGAGGTGCTGGTGAGCTGAGATCGAAACTTGGATGCTGGGTGGCTGGTGCTG
GACATCGGCTTTCGATGAGTTGGAGATCGGAGAGCTGCAGACTTTGCAGTCTTTTGCAGACTTTGCAGT
ACTGCGGCTTTTGGGTTGAAGGAAACTTGGAAGCTGGGGAGGAGGGAGGAGGGAAGCAGCTATTC
CTGGGGCTAAGCTATGCTGACTTTGTTGACTATACCGCTAAGCTGCGACTTTGCAGACACTTCTGGGGCAATTAA
AATTAGATGCGATACCCATCGCAGACACTTCTCGACAGACATCTGGGGCAATTAA
ATTTAGGAACCATGACGAGAGGTGCTGGTGAGCTGAGATCGAAACTTGGATGCTGGGTGGCTGGTGCTG
GCATCTAGCAAACGCGAGCAAAGTAGATAGTATTCCCTTGAGAGGAAACACCAACAGGCTTGAGAGGTGCT
ACAAACAAACACAGcAGcAGTCACTGCGCTTTGCAGGTGGTGATCGAGTTGGTTGATCGACTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT GG
Mouse Flk1in10 mut GATA-all
TGCATGTCAGAGATTTGTGAATTCTCTCTCTCGTTGTCAGAGTGTCGGGGAAAGGTCAGGCTCTCTG
TGCTCACTCTCTCTCTCTCTGTCAGAGTGTCGGGGAAAGGTCAGGCTCTCTG

Mouse Flk1in10 mut GATA-b,c
TGCATGTCAGAGATTTGTGAATTCTCTCTCTCTCGTTGTCAGAGTGTCGGGGAAAGGTCAGGCTCTCTG

Mouse Flk1in10 mut RBPJ/mutSOX
TGCATGTCAGAGATTTGTGAATTCTCTCTCTCTCGTTGTCAGAGTGTCGGGGAAAGGTCAGGCTCTCTG

Mouse Flk1in10 mut RBPJ
TGCATGTCAGAGATTTGTGAATTCTCTCTCTCTCGTTGTCAGAGTGTCGGGGAAAGGTCAGGCTCTCTG
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Flk1

Intron 1 enhancer

Intron 10 enhancer

Two enhancers drive Flk1 expression

Artery

Only one enhancer drives Flk1 expression

Vein

ETS
GATA
RBPJ

Activating complex

NicD

Repressive complex

Flk1

Intron 1 enhancer

Intron 10 enhancer