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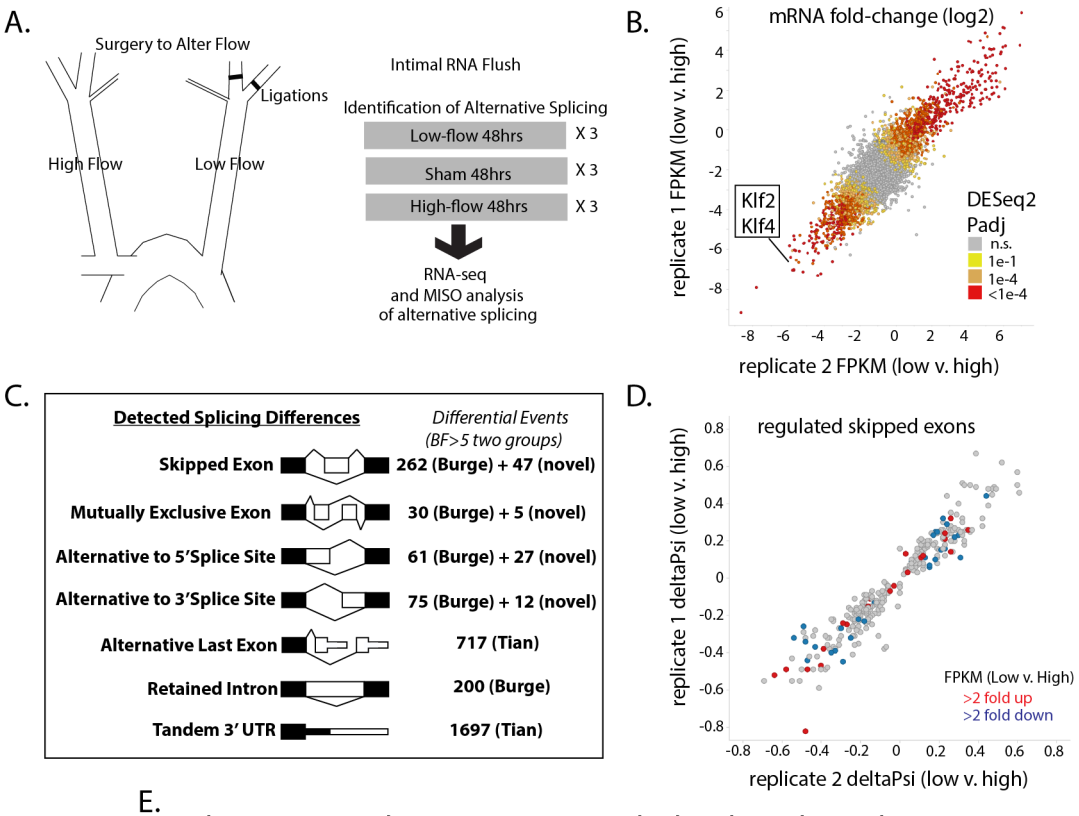
RESEARCH ARTICLE

Figures and figure supplements

Alternative RNA splicing in the endothelium mediated in part by Rbfox2 regulates the arterial response to low flow

Patrick A Murphy *et al*



E.

Rank	Process Network (GeneGO)	Regulated / Total Network	pValue
1	Transcription_mRNA processing	14/160	3.5 E-07
2	Cell adhesion_Amyloid proteins	9/195	0.005
3	Immune response_Antigen presentation	9/197	0.005
4	Cytoskeleton_Actin filaments	8/176	0.009
5	Signal transduction_ESR1-nuclear pathway	8/216	0.03
6	Cytoskeleton_Regulation of cytoskeleton rearrangement	7/183	0.03
7	Cytoskeleton_Intermediate filaments	4/81	0.05
8	Cell adhesion_Cell junctions	6/162	0.05
9	Development_Hedgehog signaling	8/254	0.06
10	Reproduction_Feeding and Neurohormone signaling	7/211	0.06

Figure 1. Exposure of the arterial endothelium to low and disturbed flow induces a program of alternative splicing. RNA from the arterial endothelium was isolated 48 hr after partial carotid ligation; from the low-flow side, the high-flow side, or sham-operated vessels. (**A**) Outline of splicing analysis. Three pools of mRNA from each condition were isolated by polyA and sequenced. (**B**) Plot showing the consistency of changes in gene expression in two independent biological comparisons of low-flow versus high-flow isolations. (**C**) Number of RNA splicing events of each category detected by MISO analysis as significantly different in two independent biological comparisons. Events were drawn from annotated databases (Burge, Tian) or from custom annotation of mapped splice junctions (novel). (**D**) Plot showing the changes in skipped-exon inclusion level (deltaPsi) between low-flow and high-flow isolations. The plot also indicates changes in transcription, highlighting exons in genes with a change in FPKM of more than 2-fold up or down. (**E**) Processes enriched in the genes with regulated skipped exons, relative to the entire set of genes expressed in the tissue with annotated skipped exons which were not significantly regulated. Padj = Adjusted P-value, BF = Bayes Factor; FPKM = Fragments Per Kilobase of transcript per Million mapped reads; CCDS = Consensus Coding DNA Sequence.

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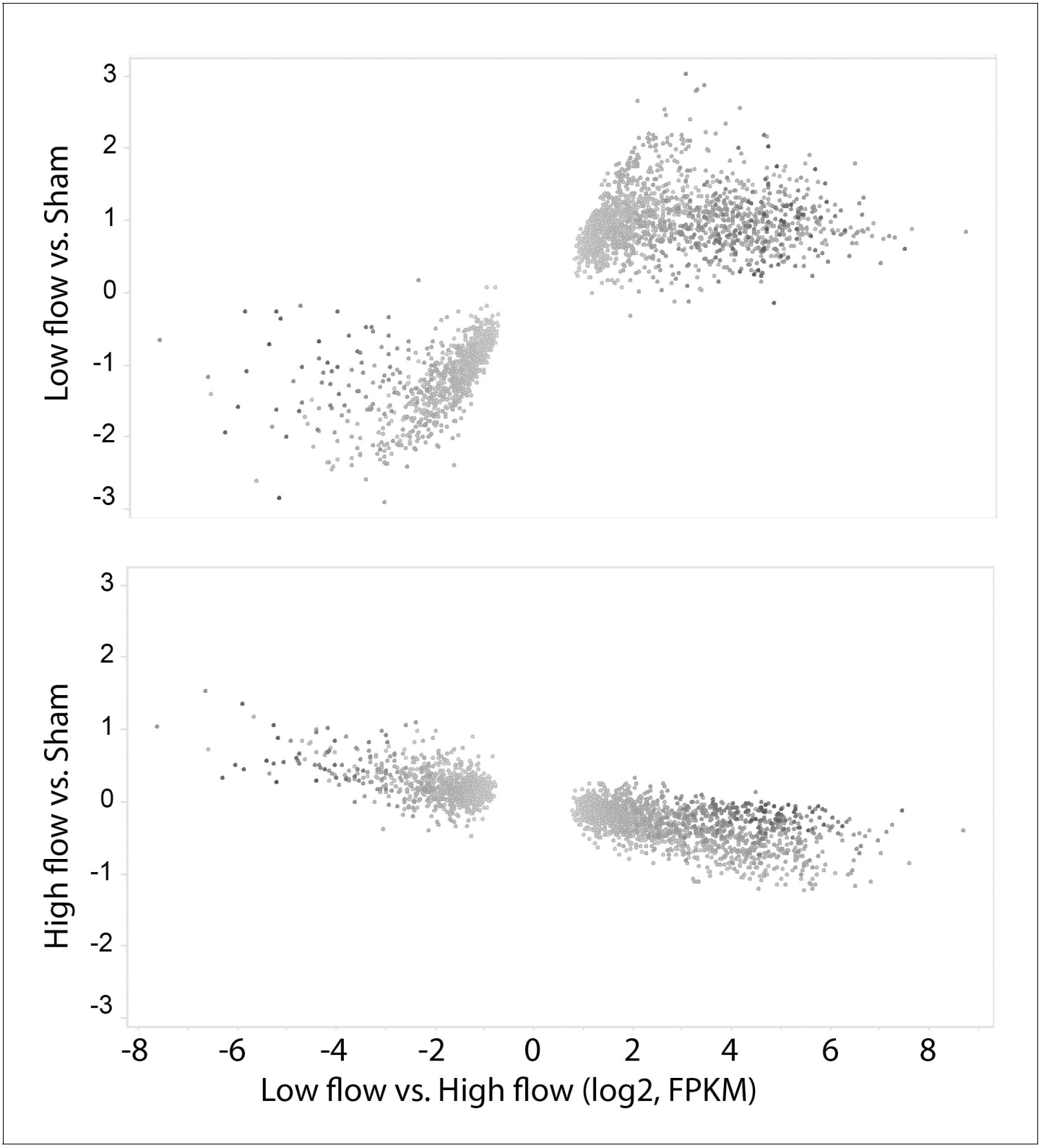

Figure 1—figure supplement 1. Gene expression changes between low-flow and high-flow intima are mainly due to low flow. Alterations in transcript level for the 2535 genes with p-values<0.0001 in the comparison of low versus high flow. Most of the changes seen in the low vs high flow comparison (X-axis) were stronger in the low vs sham comparison than in the high vs sham comparison (Y-axis). All changes are shown as log2-fold.

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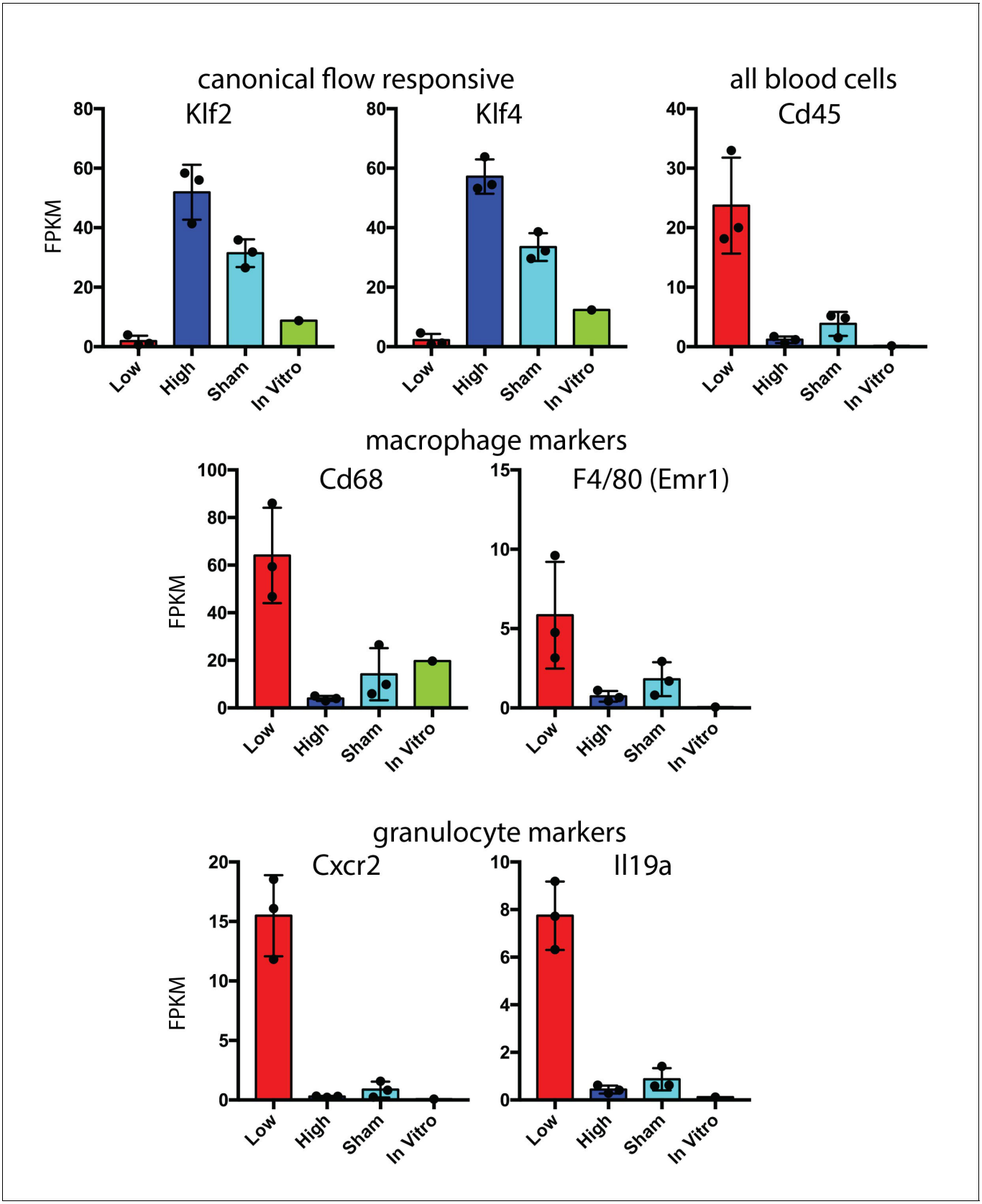

Figure 1—figure supplement 2. Hematopoietic cell recruitment within 48 hr of low and disturbed flow. Comparison of RNA-seq data from carotid intima 48 hr after the induction of low and disturbed flow. Canonical flow-responsive genes (*Klf2* and *Klf4*) and markers of recruited immune cells (*CD45*,

Figure 1—figure supplement 2 continued on next page

Figure 1—figure supplement 2 continued

Cd68, *F4/80*, *Cxcr2* and *Il19a*) are shown. Each point represents pooled RNA from multiple intimal preparations. In vitro is a single isolated and sorted cell line from aortic endothelium based on eGFP expression (*Cdh5(PAC)-CreERT2; mTmG*).

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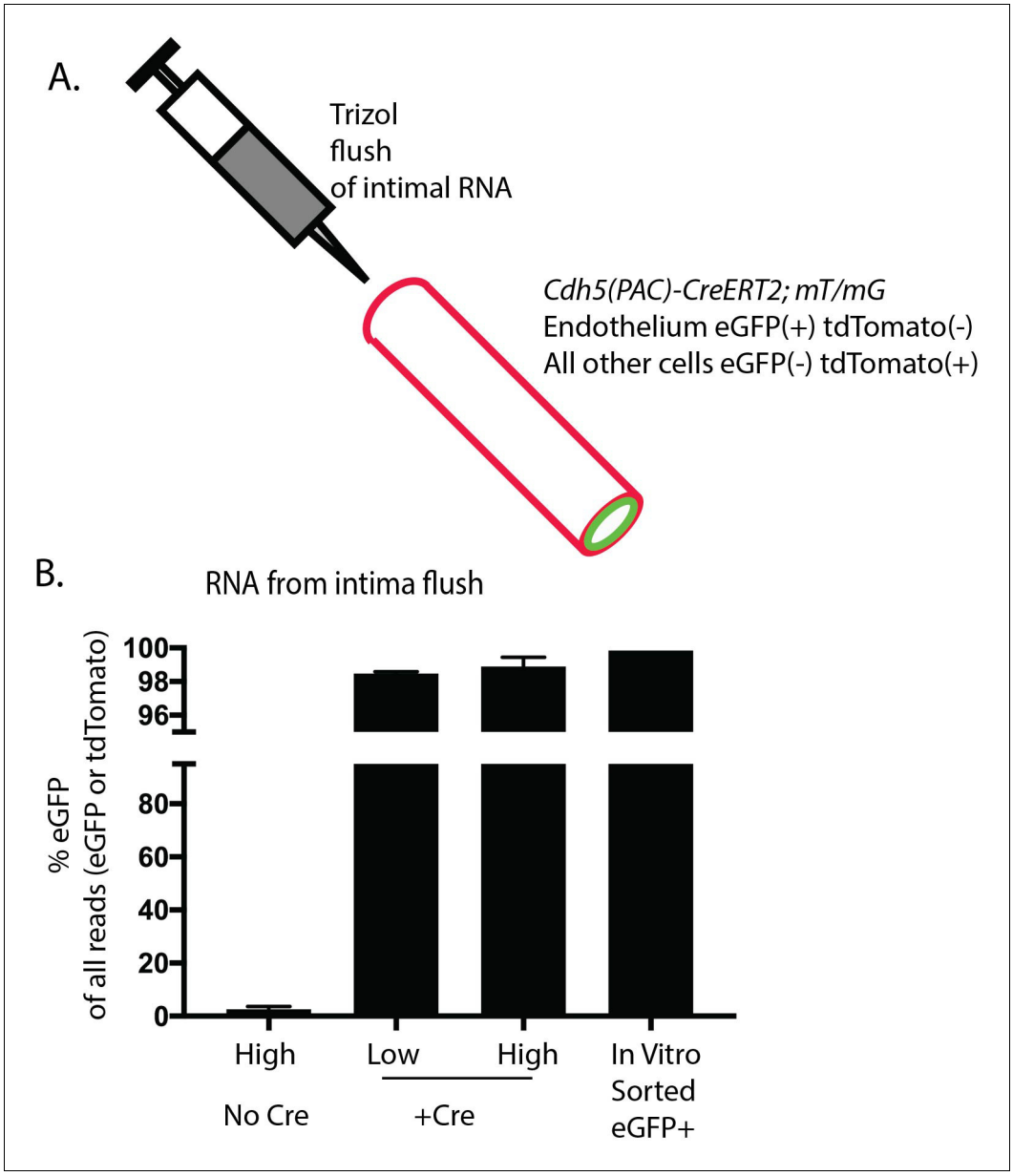

Figure 1—figure supplement 3. Enrichment of endothelial RNA demonstrated by lineage markers in mT/mG mice. The *mT/mG* mice constitutively express *tdTomato* in all cells from a CMV enhancer/chicken beta-actin core promoter (pCA). Upon Cre activity, in this case driven by the endothelial-specific *Cdh5(PAC)-CreERT2*, the *tdTomato* is excised, and a downstream *eGFP* is expressed instead. Since *tdTomato* is expressed in all non-endothelial cells and *eGFP* is expressed in all endothelial cells, the ratio may be used as an indicator of endothelial to non-endothelial mRNA levels in the isolate. The comparison of RNA-seq reads assigned to *eGFP* or *tdTomato* from *Cdh5(PAC)-CreERT2; Rbfox2 ff; mT/mG* (N = 2 pools) mice or *Rbfox2 ff; mT/mG* mice (N = 2 pools) is shown. Cultured and FACs-purified eGFP +cells from *Cdh5(PAC)-CreERT2; Rbfox2 ff; mT/mG* mouse aorta is also shown (N = 1).

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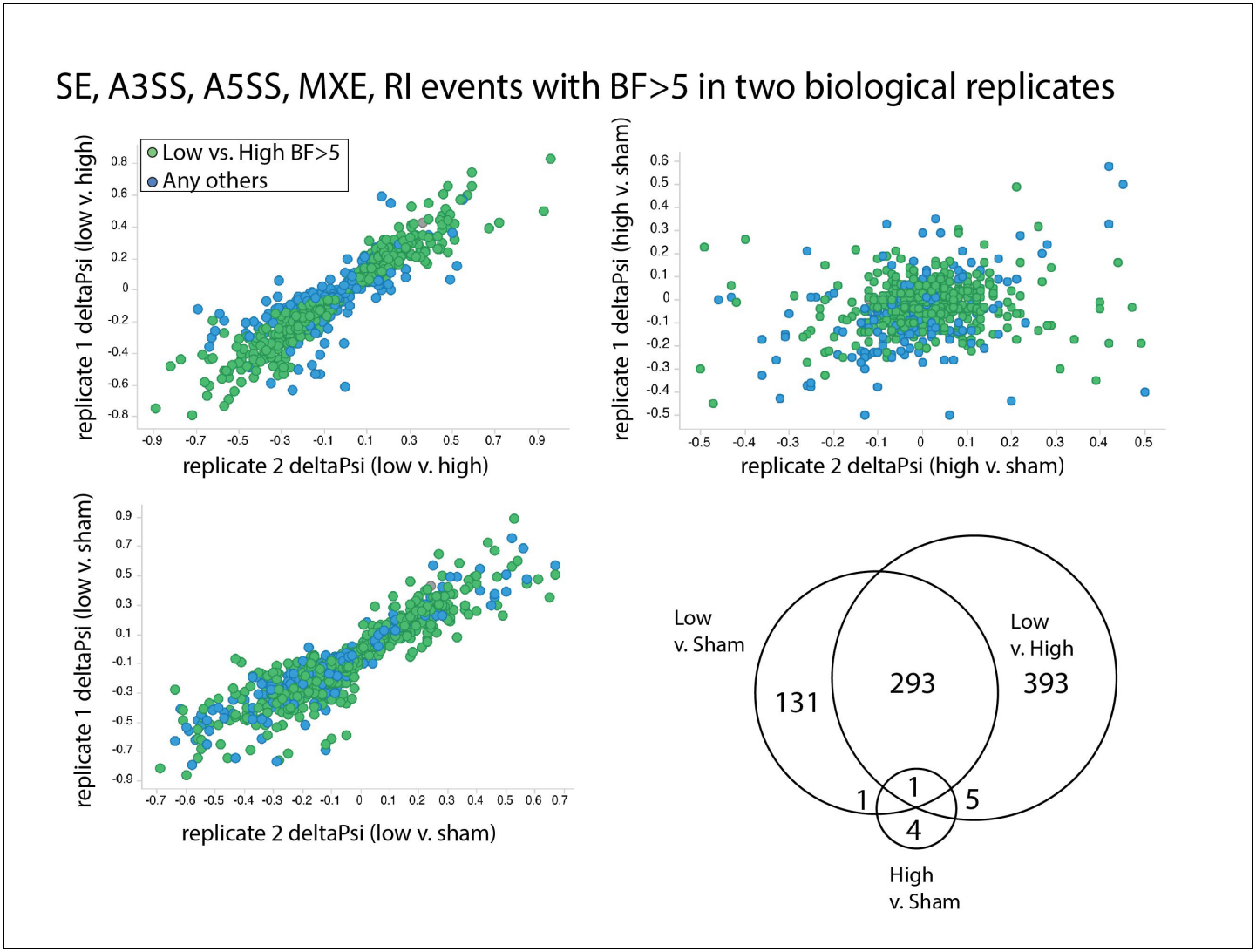

Figure 1—figure supplement 4. Splicing changes are primarily induced by low flow rather than high flow. Comparison of the change in inclusion frequency by MISO for individual splicing events of the SE, MXE, A3SS, A5SS and RI classes. In green are the events presented in ***Figure 1C***. In blue are all other events with BF >5 in two independent biological comparisons of low flow versus sham, or with BF >5 in two independent biological comparisons of high flow versus sham. Then Venn diagram shows the overlap in these events found to be significant in each of the independent comparisons.

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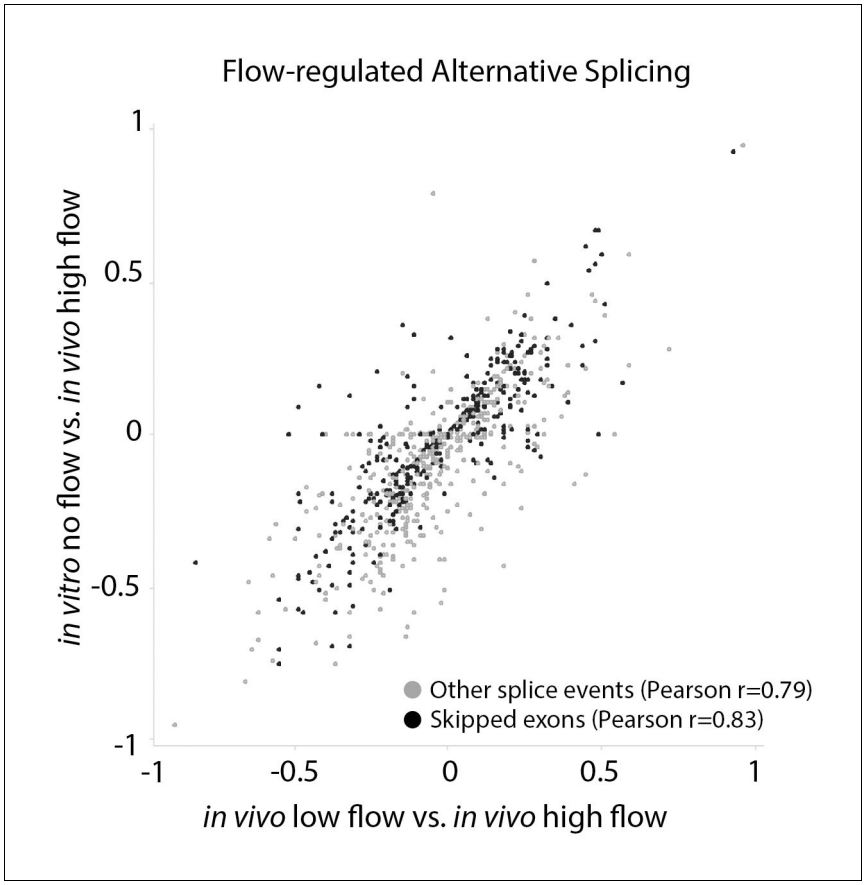

Figure 1—figure supplement 5. Arterial cells cultured in vitro replicate splicing changes observed under low and disturbed flow in vivo. Comparison of the change in inclusion frequency by MISO for individual splicing events of the classes shown (by color code on bottom), relative to high-flow arterial endothelium in vivo. Other splice events include A3SS, A5SS, MXE and RI. Only flow-regulated alternative splicing events are shown. In vitro cells are primary *Cdh5(PAC)-CreERT2; mT/mG* cells from the aorta, isolated as described in Materials and methods, cultured in static conditions and sorted by FACs for eGFP+ before RNA isolation. Splicing events in these cells were compared to high-flow carotid artery intimal isolate. In vivo low-flow is the comparison of the low-flow intimal isolate to the high-flow intimal isolate. Pearson correlation indicates the correlation between the change in inclusion observed in low-flow activated endothelial cells in vivo versus low-flow activation of endothelial cells in vitro.

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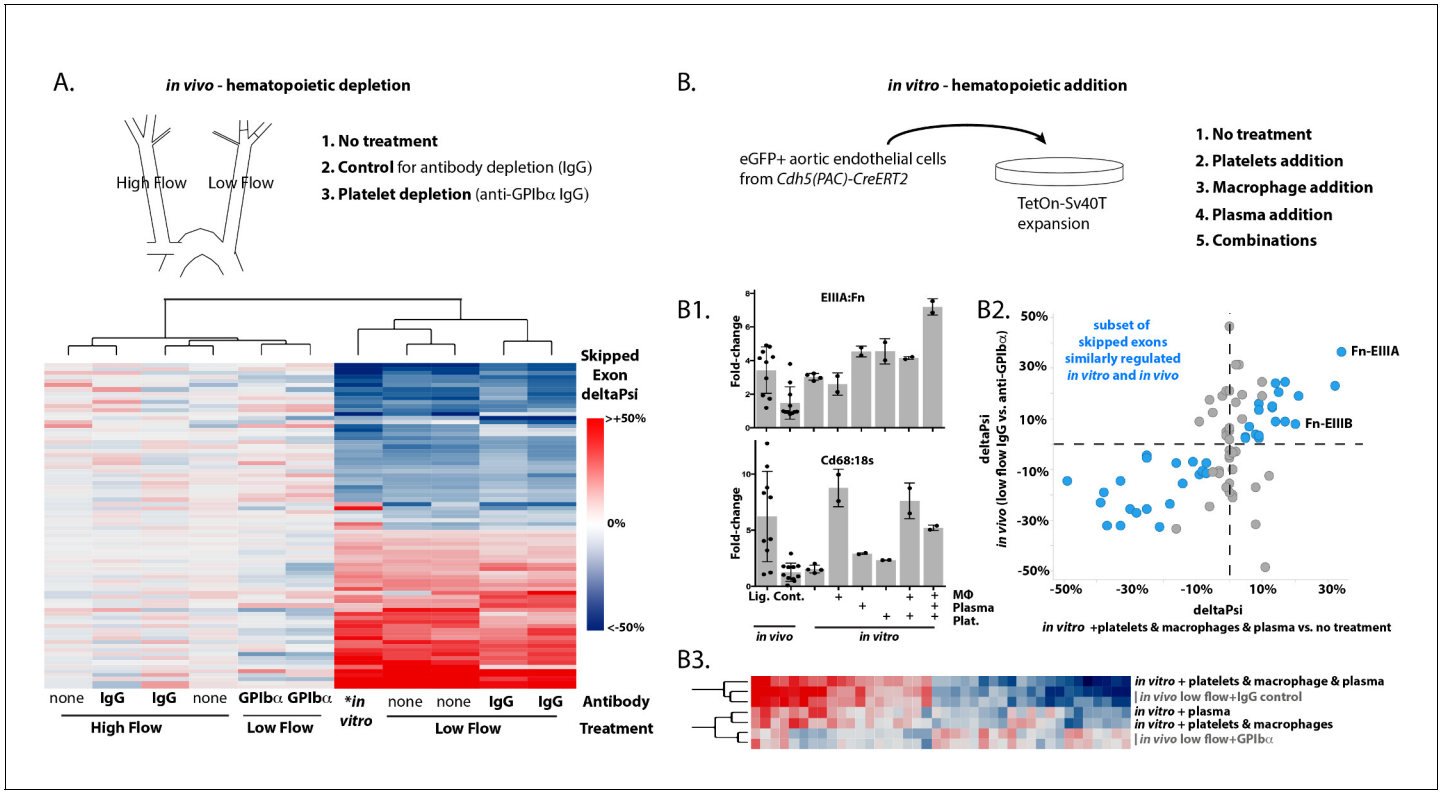

Figure 2. Platelets and macrophages regulate a subset of flow-regulated skipped-exon events. (**A**) Effects of platelet depletion on splicing patterns in vivo (see also 2–2). Clustered heat map showing the change in skipped-exon inclusion frequency relative to high-flow contralateral arteries in biological replicates of untreated arteries (from data set created in ***Figure 1***) or platelet-depleted (anti-GPIbα) or IgG control-treated (IgG) arteries. The data shown are for the platelet-dependent subset of all flow-regulated skipped exons (SE; 80/292). IgG and anti-GPIbα (low flow) are relative to average IgG (high flow). No antibody treatment in vitro and in vivo low flow are relative to average in vivo high flow. (**B**) Effects of platelet, macrophage and plasma addition to endothelial cells in vitro. (**B1**) Bar graphs showing in vitro changes in EIIIA inclusion frequency and Cd68 macrophage marker expression with the different treatments of conditionally immortalized aortic endothelial cells, relative to in vivo low-flow and high-flow samples. (**B2**) Plot showing the in vitro regulation of platelet-regulated skipped-exon events (40/80), in isolated and conditionally immortalized aortic endothelial cells. (**B3**) Clustered heat map, showing the change in skipped exon inclusion frequency in in vivo biological replicates of low-flow arteries with or without platelet depletion (in vivo low-flow+ IgG control, +GPIba) or conditionally immortalized aortic endothelial cells with the addition of the indicated cells or 10% plasma.

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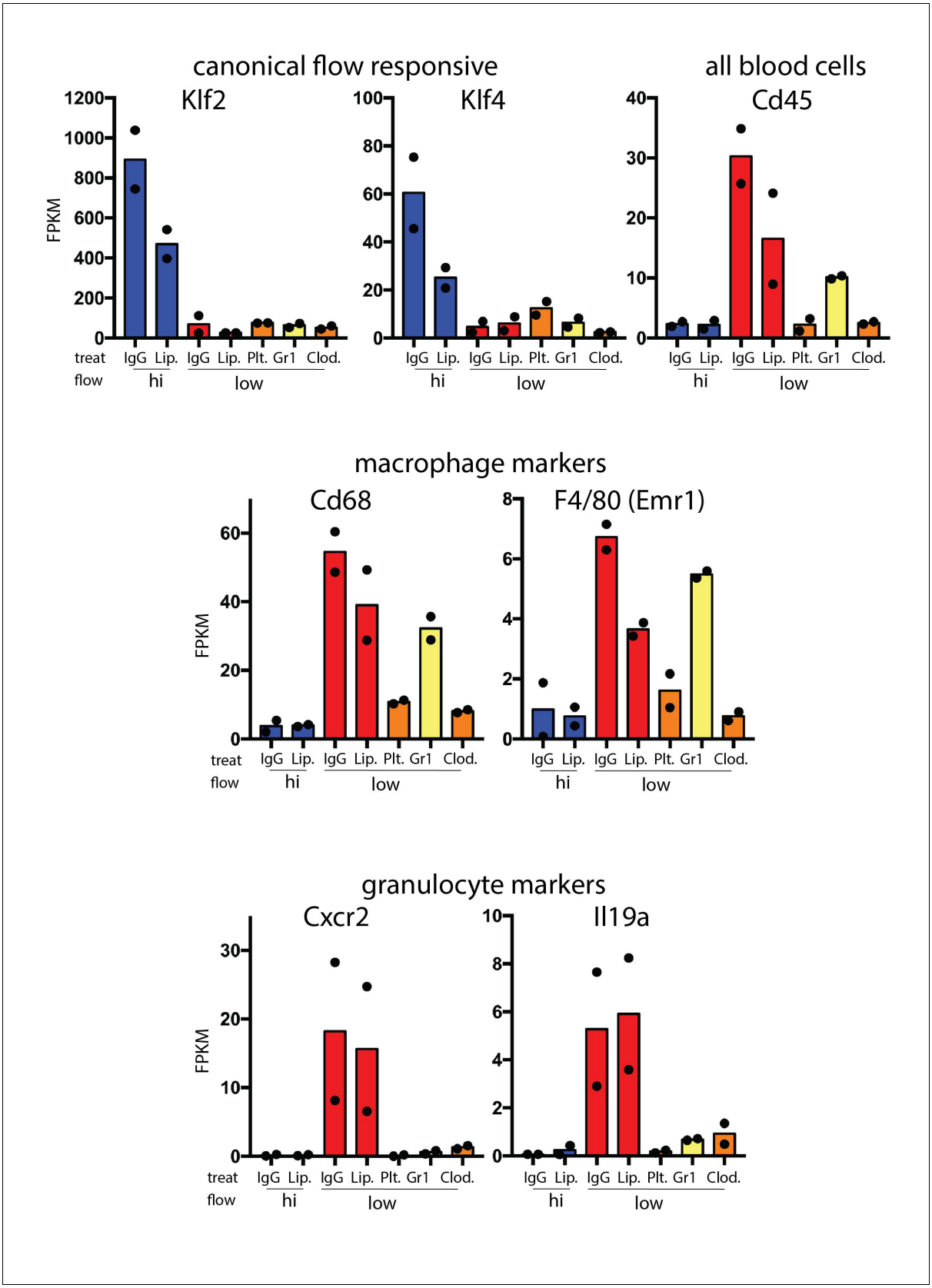

Figure 2—figure supplement 1. Hematopoietic cell depletion in aortic intima 48 hr after induction of low and disturbed flow. Comparison of RNA-seq data from carotid intima 48 hr after induction of low and disturbed flow, or in the contralateral arteries. Each point represents pooled RNA from

Figure 2—figure supplement 1 continued on next page

Figure 2—figure supplement 1 continued

multiple intimal preparations (N = 2 pools per condition, each a pool of 3–5 arteries). IgG is a non-specific antibody control for platelet (Plt.) and granulocyte (Gr1)-depleting antibodies. PBS liposome (Lip.) is a control for macrophage-depleting clodronate liposomes (Clod.).

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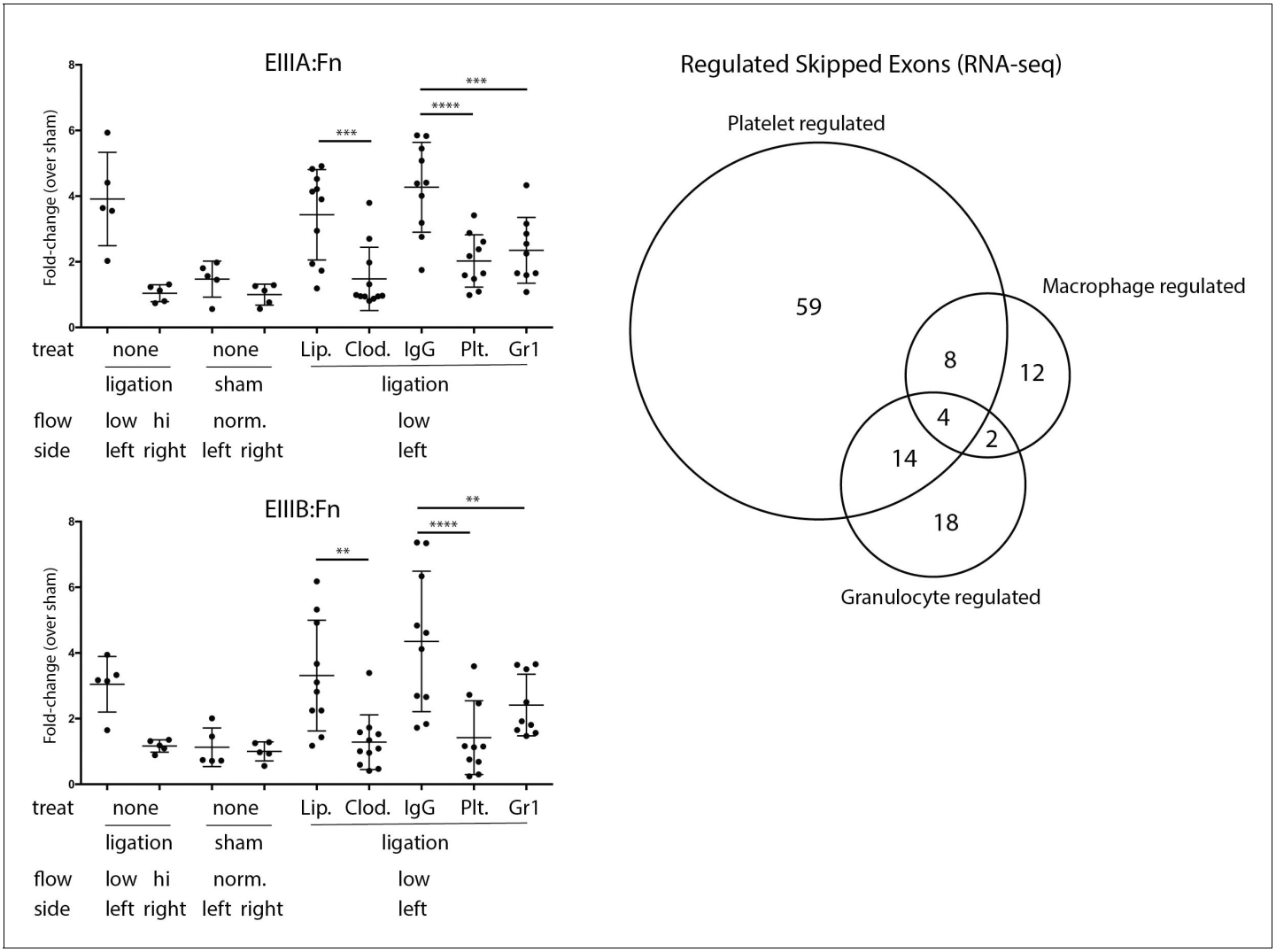

Figure 2—figure supplement 2. In vivo depletions of individual hematopoietic cell populations and their effects on Fn-EIIIA and –EIIIB inclusion. Quantitative PCR results of low-flow carotid arteries isolated from mice treated with anti-Gr1 (granulocytes), anti-GPIbα (platelets), clodronate liposomes (macrophages), or controls (IgG for antibody treatments and PBS liposomes for clodronate liposomes). Change in inclusion frequency is shown as fold change relative to sham-operated control contralateral artery at 48 hr. Significance of the differences by Sidak's multiple comparison test are shown (**p<0.01, ***p<0.001, ****p<0.0001). Venn Diagram shows the low-flow events significantly regulated in vivo by platelets, macrophages or granulocytes, as defined in methods.

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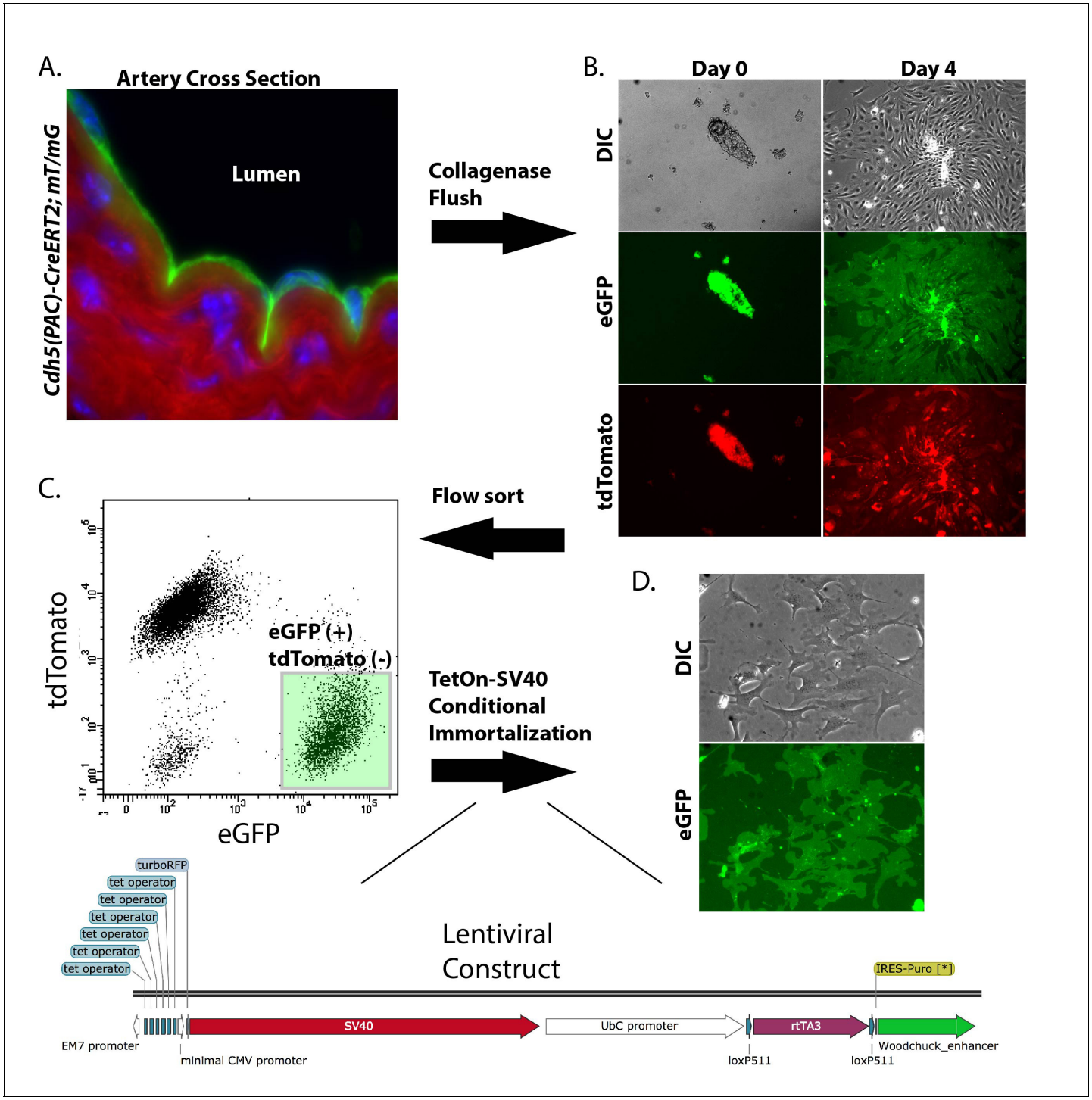

Figure 2—figure supplement 3. Isolation and conditional immortalization of aortic endothelial cells. Endothelial cells were marked by induction of the mT/mG reporter in *Cdh5(PAC)-CreERT2; mT/mG* mice (**A**), isolated by collagenase flush and cultured (**B**), sorted on the mT/mG markers (**C**) and then immortalized by TetOn-SV40T (**D**).

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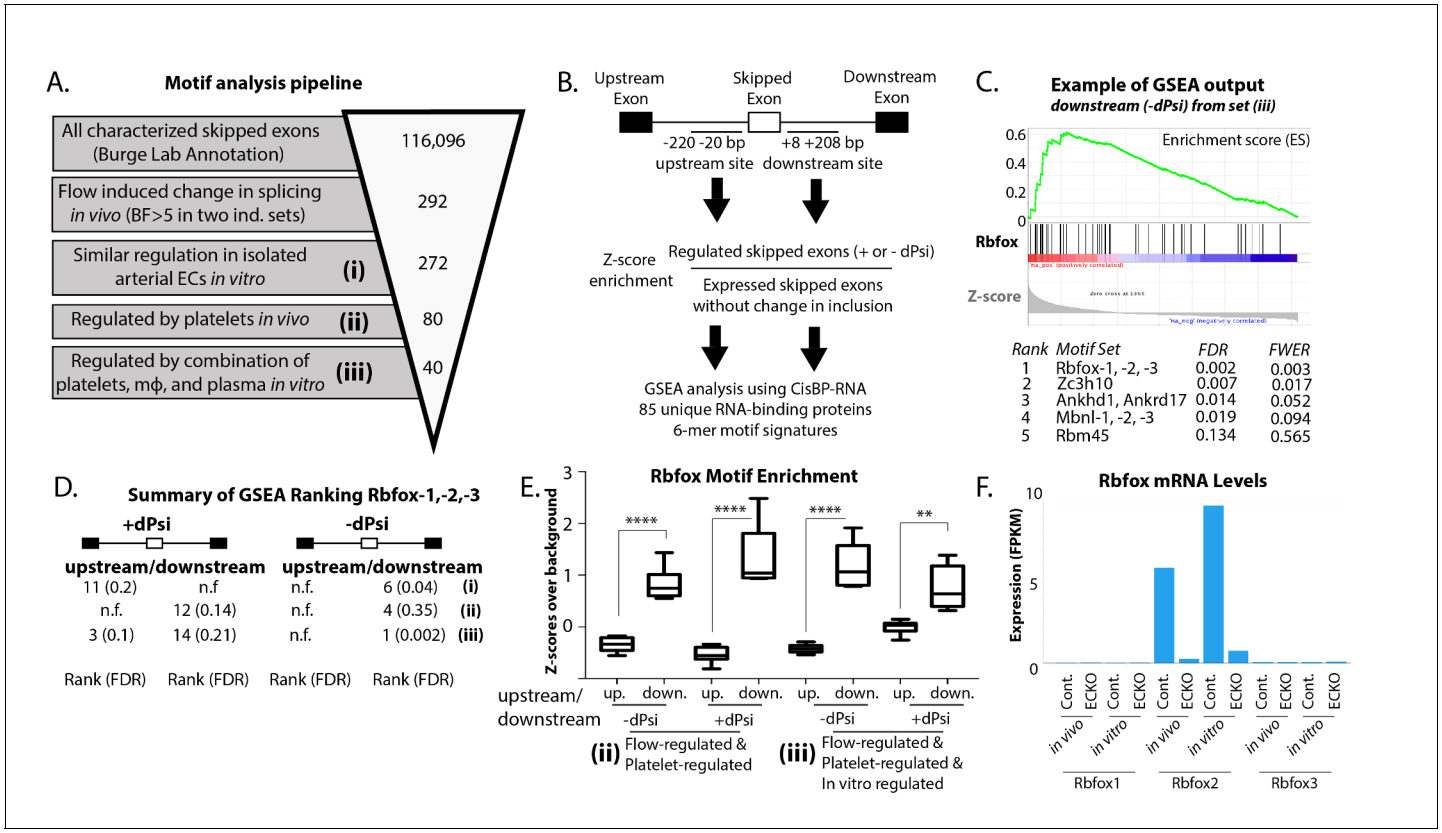

Figure 3. Enrichment of an Rbfox motif in the platelet/macrophage-regulated subset identified in vivo and in vitro. (**A**) Motif analysis pipeline, showing the number of potentially regulated skipped-exon events, and those passing each filter. (**B**) Events were separated into those with increased or decreased inclusion, and then the sequences of the upstream and downstream 200 bp flanking regions were isolated, resulting in four regions of analysis. 6-mer motifs in each region were assessed for enrichment relative to exons expressed in six matched background sets of skipped exons not regulated by flow. GSEA was used to identify splicing factor 'fingerprints' among these enriched motifs, using the motif-binding preference defined by the CisBP-RNA database (Ray et al.). (**C**) Example of enrichment of Rbfox binding motif in GSEA analysis of 85-unique RNA-binding protein signatures against flanking intron sequences of flow-regulated exons. Plot shows enrichment of Rbfox family motifs among the motifs most enriched above background in the downstream flanking region of exons regulated in vivo and in vitro (set iii). (**D**) Enrichment of the Rbfox2 motif in each of the sets of biologically defined regulated exons (i-iii) within the upstream and downstream flanking regions of exons with either increased or decreased inclusion. (**E**) Plot showing the average z-score enrichment of the top 5 Rbfox in vitro defined motifs in the flanking regions of the in vivo regulated exon set (80 SE) and the in vitro regulated subset (40 SE), relative to the six matched background sets (N = 6 per bar). (**F**) Transcript levels of Rbfox family of proteins in the carotid artery in vivo and in isolated aortic endothelial cells in vitro, with or without the deletion of *Rbfox2* by *Cdh5(PAC)-CreER* (EC-KO). FPKM = Fragments Per Kilobase of transcript per Million mapped reads. FDR; false-discovery rate; n.f.; not found. p<0.0001 (****), p<0.01 (**).

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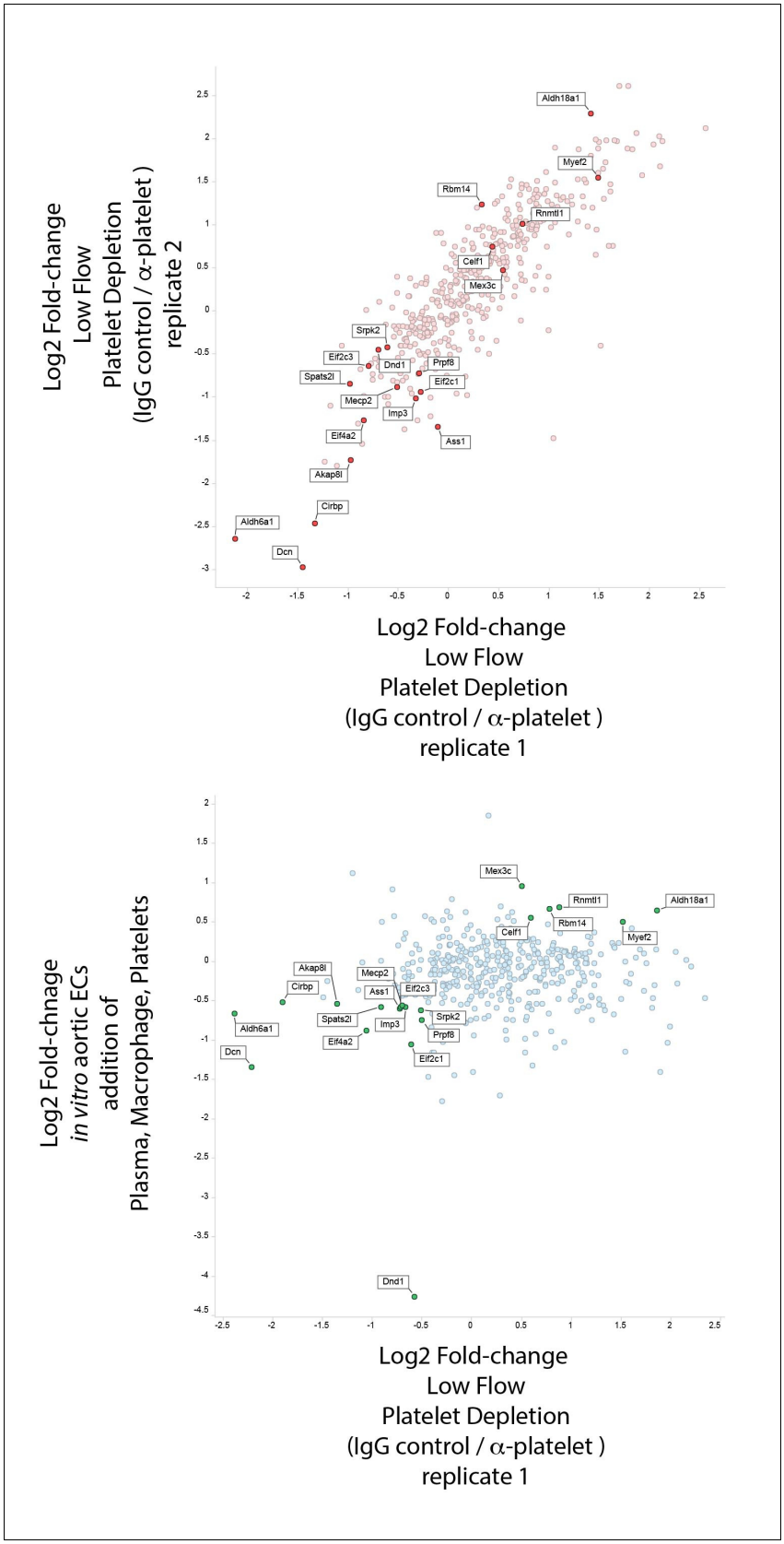

Figure 3—figure supplement 1. No obvious candidate splice factors from differential expression under altered flow conditions. Differential expression of RNA-binding proteins under conditions with altered splicing. Factors

Figure 3—figure supplement 1 continued on next page

Figure 3—figure supplement 1 continued

highlighted had consistent differences of <−0.5 or >0.5 in both sets of data. Only *Dcn* (*decorin*) showed differences of <-1 in both. RNA-binding proteins with FPKM <0.5 in the in vivo data sets were filtered out.

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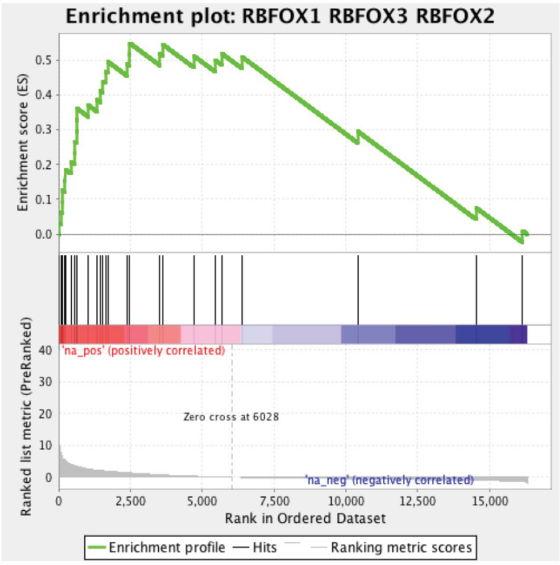
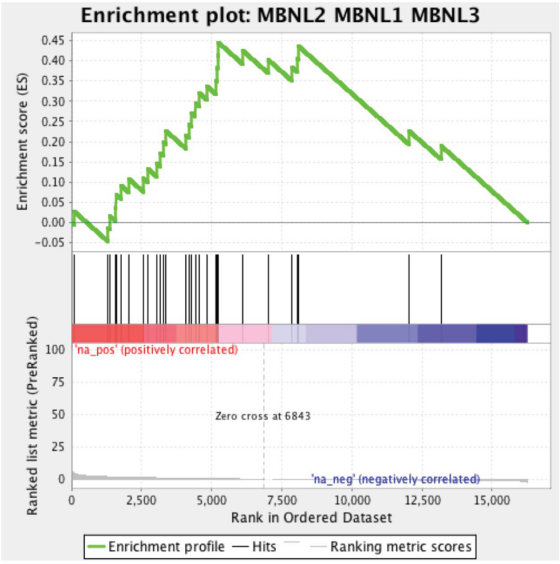
A. Rbfox- 5′ SS of increased inclusion exons



	GS follow link to MSigDB	GS DETAILS	SIZE	ES	NES	NOM p-val	FDR q-val	FWER p-val	RANK AT MAX	LEADING EDGE
1	RBFOX1 RBFOX3 RBFOX2	Details ...	30	0.55	3.65	0.000	0.000	0.000	2481	tags=70%, list=15%, signal=82%
2	EIF2S1	Details ...	30	0.54	3.51	0.000	0.000	0.000	6378	tags=93%, list=39%, signal=153%
3	RBM4B ENSMUSG00000056951	Details ...	27	0.46	2.83	0.000	0.000	0.000	8757	tags=100%, list=54%, signal=215%
4	PPRC1	Details ...	27	0.42	2.55	0.000	0.001	0.005	8348	tags=93%, list=51%, signal=189%
5	CELF3	Details ...	30	0.35	2.35	0.000	0.006	0.026	6795	tags=77%, list=42%, signal=131%
6	ELAVL1	Details ...	30	0.36	2.33	0.000	0.006	0.029	5597	tags=70%, list=34%, signal=106%

B. Mbnl - 5′ SS of increased inclusion exons



	GS follow link to MSigDB	GS DETAILS	SIZE	ES	NES	NOM p-val	FDR q-val	FWER p-val	RANK AT MAX	LEADING EDGE
1	MBNL2 MBNL1 MBNL3	Details ...	30	0.45	2.90	0.000	0.000	0.000	5234	tags=77%, list=32%, signal=113%
2	SYNCRIP	Details ...	30	0.34	2.22	0.000	0.030	0.062	8044	tags=83%, list=49%, signal=165%
3	PTBP1 PTBP2 ROD1	Details ...	30	0.32	2.12	0.002	0.041	0.117	7246	tags=77%, list=45%, signal=138%
4	CELF3	Details ...	30	0.29	1.94	0.006	0.083	0.290	6594	tags=70%, list=41%, signal=118%
5	PCBP2	Details ...	30	0.30	1.92	0.008	0.071	0.304	5948	tags=67%, list=37%, signal=105%
6	CELF4	Details ...	30	0.26	1.76	0.021	0.150	0.605	6594	tags=67%, list=41%, signal=112%
7	RBM46	Details ...	30	0.26	1.73	0.020	0.156	0.677	8181	tags=77%, list=50%, signal=154%
8	PPRC1	Details ...	26	0.28	1.68	0.025	0.173	0.758	11749	tags=100%, list=72%, signal=360%

Figure 3—figure supplement 2. Positive control sets to confirm the motif-based identification of regulated events in vitro and in vivo by their CisBP-RNA fingerprint in GSEA. GSEA plots showing enrichment of top CisBP-RNA motifs for the indicated splice factors near the skipped exons of the events regulated in the published gene sets (Rbfox2 shRNA in ES cells in vitro from Jiangi M et al; Mbnl1 in heart of KO mice from Wang ET et al.).
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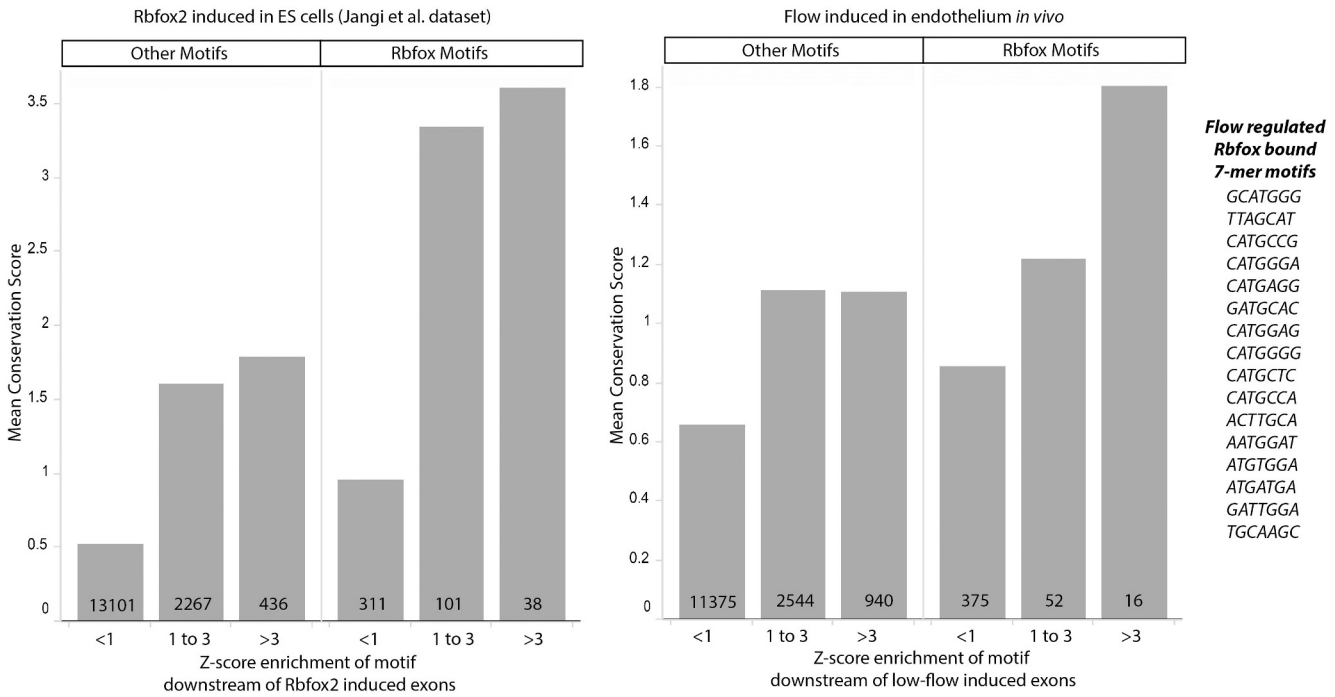

Figure 3—figure supplement 3. Conservation of Rbfox2 motifs enriched near flow-regulated skipped exons. Plot showing the average conservation score (y-axis) and z-score (x-axis) for individual motifs near induced skipped exons in the published positive-control set (Rbfox2 regulated, Jangi et al. Genes and Development 2015) and the flow-induced set of skipped exon described here. Rbfox Motifs and Other Motifs are defined by Rbfox2 binding in vitro in the CisBP-RNA database (z-score >1 in CisBP-RNA). Mean conservation score on the Y-axis is derived from PhyloP vertebrate score at for each nucleotide in the given 7-mer motif adjacent to the regulated skipped exons. Z-score enrichment on the X-axis bins motifs into strongly (z-score >3), moderately (z-score 1 to 3) and weakly (z-score <1) enriched in the downstream region (5'ss) of induced skipped exons, relative to background sets of unregulated skipped exons. Number of motifs in each column are shown at the bottom of the bar. Motifs are shown for the far-right column of flow-induced (Rbfox2 motif z-score >1 in CisBP-RNA, and z-score enrichment >3 downstream of flow induced skipped exons).

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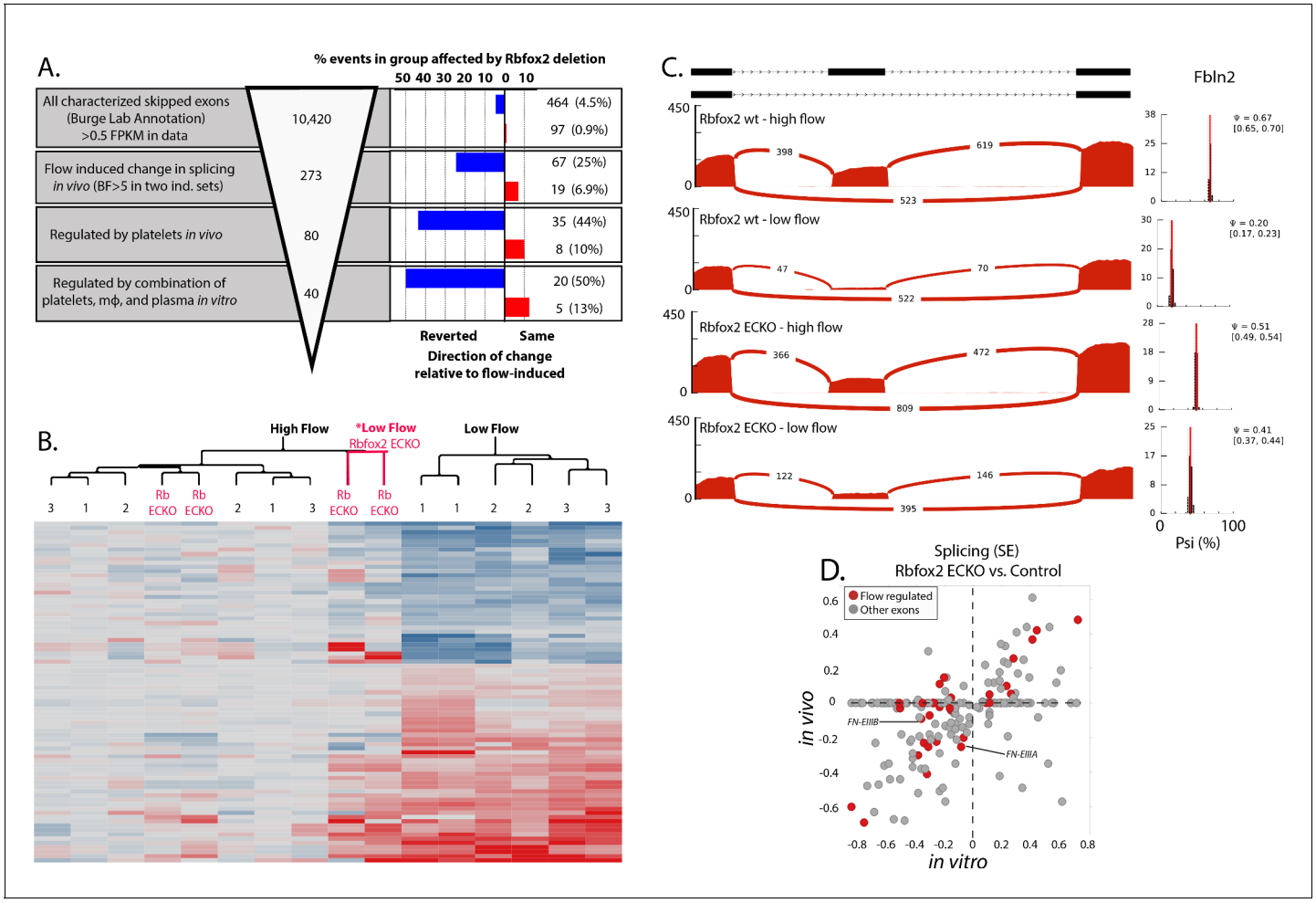

Figure 4. Endothelial Rbfox2 deletion affects many of the flow-regulated splicing events. (**A**) Showing the percentage of each group of skipped exons regulated by endothelial deletion of Rbfox2 (BF >5) in any comparison and regulated similarly in both comparisons of Rbfox2 wt low-flow vs. Rbfox2 EC-KO low-flow – either consistent with the flow-induced change in splicing (red, 'same') or against the direction of the flow-induced change in splicing (blue, 'reverted'). (**B**) Heat map showing the clustering of flow-regulated splicing events reverted by Rbfox2 deletion (67 of the 273 events consistently regulated between 48 hr and 7 days). 1 = C57 wild-type mice, 48 hr data set; 2 = C57 wild-type mice, IgG control 48 hr data set; 3 = Rbfox2 wt control (i.e. no Cre), 7 days data set; Rb EC-KO = Rbfox2 EC-KO 7 days data set. (**C**) Effect of endothelial *Rbfox2* deletion on flow-mediated regulation of inclusion of *Fbln2* alternative exons. (**D**) Plot shows the change in splicing of skipped exons (SE) following *Rbfox2* deletion from carotid artery intima in vivo (under low flow) or in isolated primary aortic endothelial cells in vitro. SE from the *Rbfox2* regulated set of events, in genes expressed >FPKM 1 in in vitro and in vivo sets. SE also regulated by a change in flow are highlighted.

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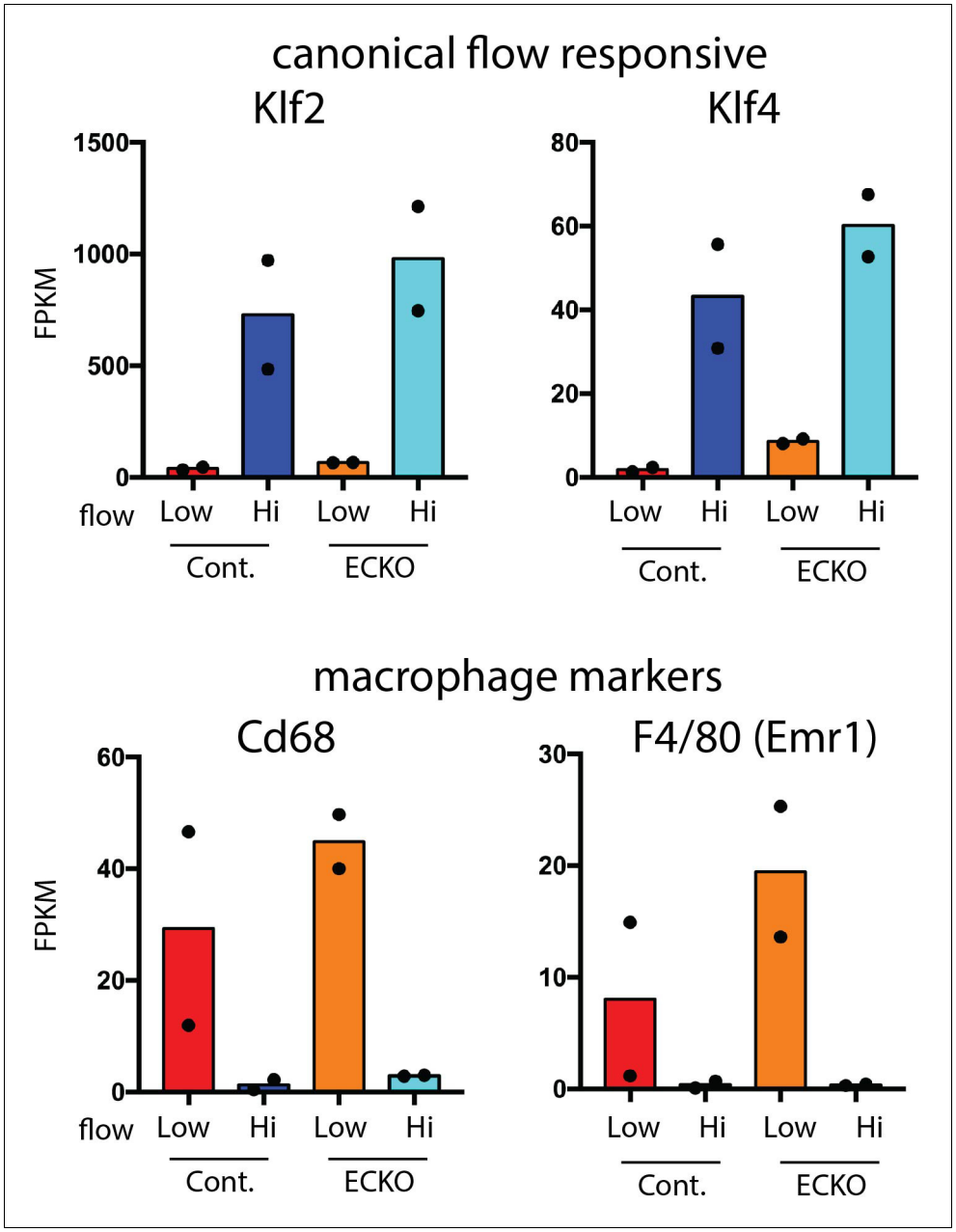

Figure 4—figure supplement 1. Rbfox2 deletion does not cause a reduction in markers of recruited platelets or macrophages. Results of RNA-seq analysis of selected transcripts of intimal flush of the ligated low-flow side or the contralateral high-flow side at 7 days in Rbfox2 EC-KO mice or littermate controls.

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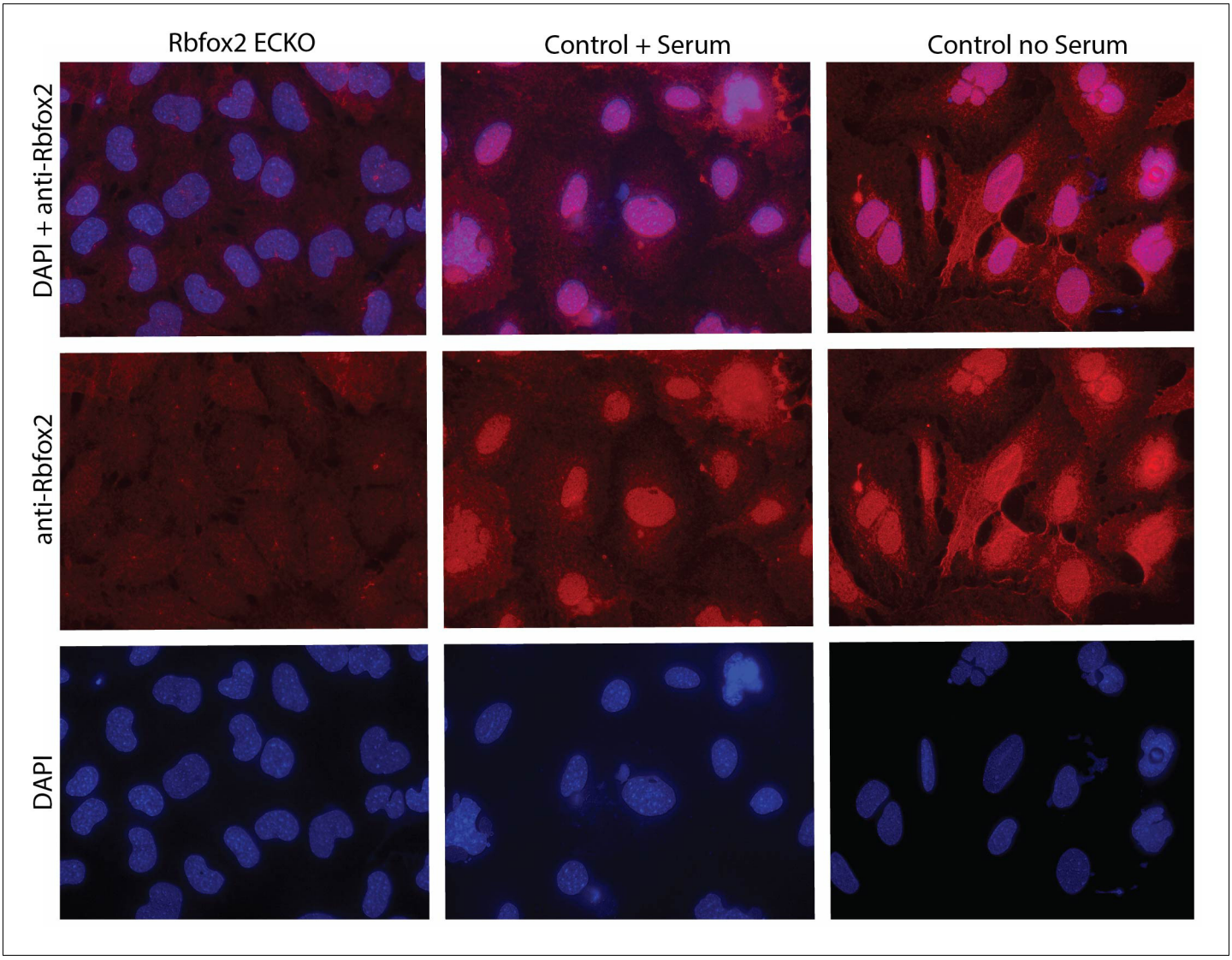

Figure 4—figure supplement 2. Rbfox2 localization under serum stimulation in mouse aortic endothelial cells. Immunofluorescence staining for Rbfox2 protein in aortic endothelial cells in culture under conditions (high density + FBS) which induce *EIIIA* and *EIIIB* inclusion versus conditions which do not (high density no FBS).

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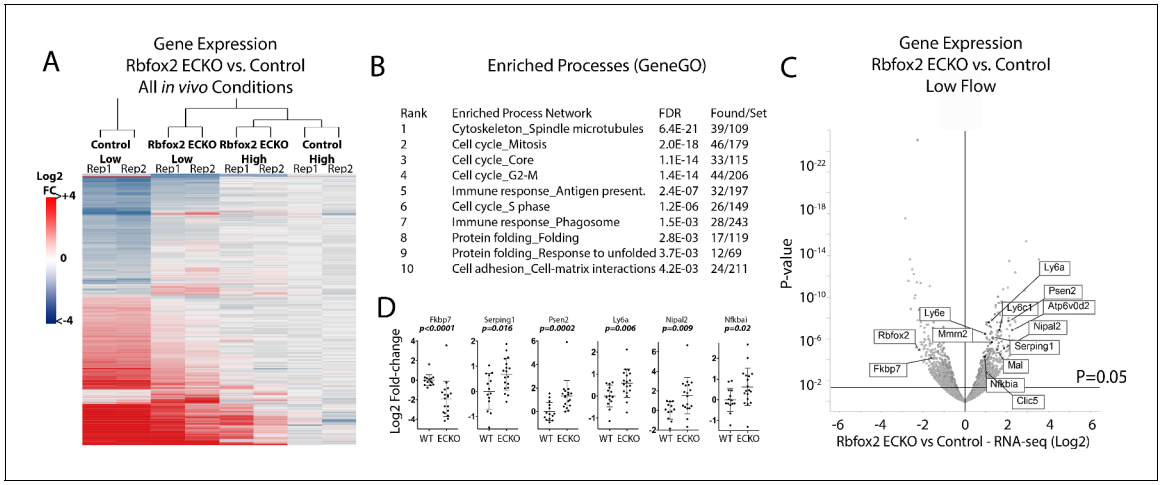

Figure 5. Endothelial Rbfox2 deletion suppresses low-flow transcript response in the arterial intima. (**A**) Clustered heat map of the genes with adjusted p-values<0.05 shows the change in expression relative to contralateral controls at 7 days after the change in flow in the indicated genotypes (N = 641). (**B**) Enriched terms among the genes regulated by Rbfox2 deletion. (**C**) Volcano plot showing DESeq2 calculated p-values and log2 fold changes in genes expressed in the arterial intima at 7 days of arteries exposed to low and disturbed flow, with or without deletion of *Rbfox2*. Genes selected for qPCR in single arteries are shown. (**D**) Results of individual carotid artery qPCR for the genes indicated. Log2 fold-changes are relative to control, p values are from Mann-Whitney test (N = 14 control and N = 18 Rbfox2 EC-KO).

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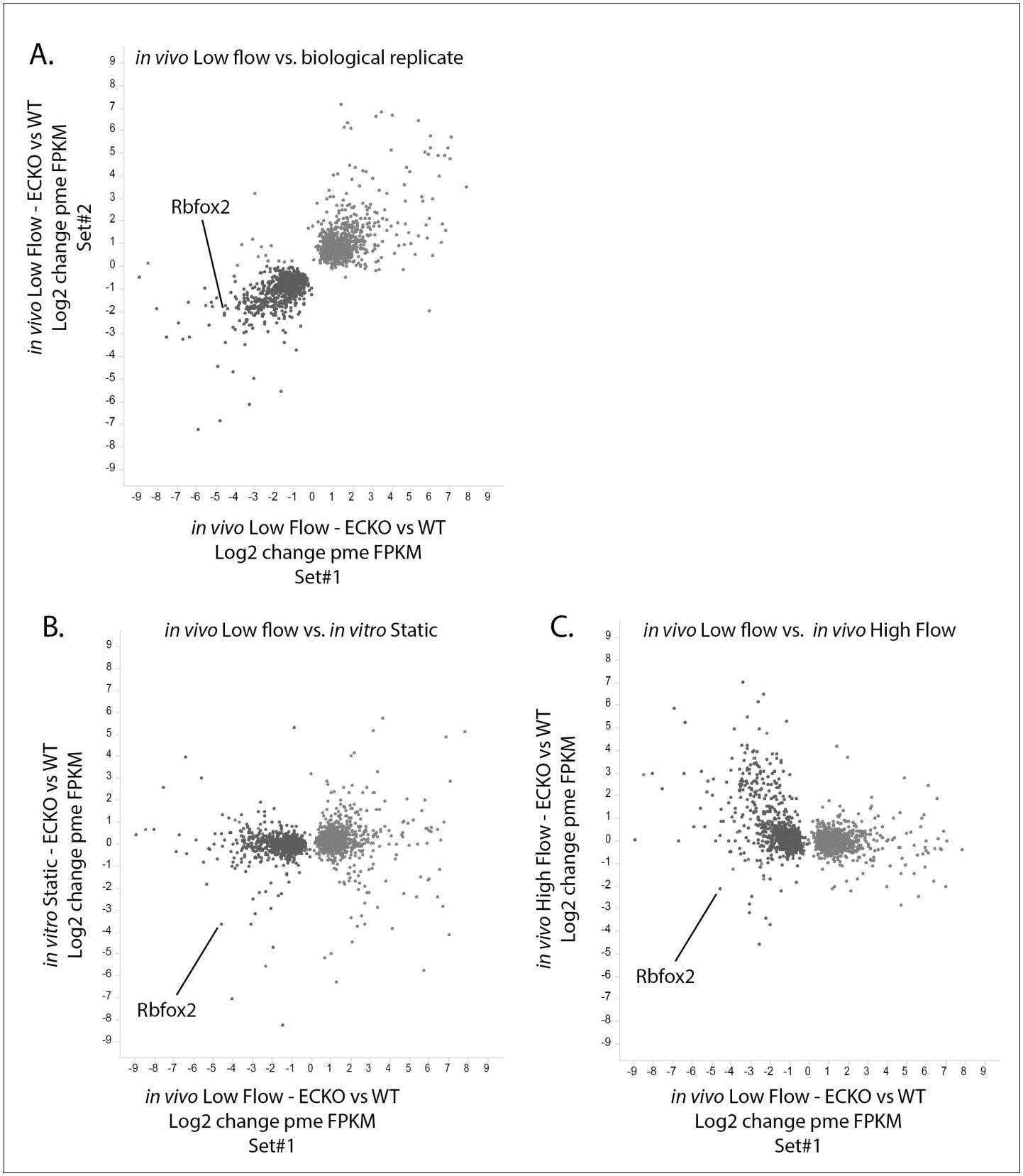

Figure 5—figure supplement 1. Rbfox2-dependent transcriptional response found in vivo is specific to the low-flow arterial intima. Change in expression in the low-flow intima of Rbfox2 EC-KO mice versus littermate control mice (X-axis) plotted against the Y-axis showing (**A**) a biological

Figure 5—figure supplement 1 continued on next page

Figure 5—figure supplement 1 continued

replicate of the same comparison, (**B**) a comparison of isolated and cultured (7 days) aortic endothelial cells from Rbfox2 EC-KO mice versus littermate control mice, or (**C**) a comparison of the contralateral high- flow intima of Rbfox2 EC-KO mice versus littermate control mice. Gene expression (pme FPKM) was calculated by RSEM, and only genes with p-values of <0.05 (DESeq2) between Rbfox2 EC-KO and control under low-flow conditions are shown.

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