Zyxin Mediation of Stretch-Induced Gene Expression in Human Endothelial Cells

Agnieszka Wójtowicz,* Sahana Suresh Babu,* Li Li, Norbert Gretz, Markus Hecker, Marco Cattaruzza

Rationale: Prolonged exposure to enhanced stretch, such as in hypertension, triggers endothelial dysfunction, a hallmark of pathological vascular remodeling processes. Despite its clinical relevance, little is known about stretch-induced gene expression in endothelial cells.

Objective: Here, we have characterized a new stretch-inducible signaling pathway and the subsequent changes in endothelial gene expression in response to stretch.

Methods and Results: Using human primary endothelial cells, we observed that the protein zyxin translocates from focal adhesions to the nucleus solely in response to stretch. There, it orchestrates complex changes in gene expression by interacting with a novel cis-acting element found in all zyxin-regulated genes analyzed so far. By way of DNA microarray pathway analyses, stretch-induced changes in endothelial cell gene expression were systematically explored, revealing that zyxin mainly regulates proinflammatory pathways.

Conclusions: Stretch appears to be an important factor in the development of endothelial dysfunction with zyxin as a potential therapeutic target to interfere with these early changes in endothelial cell phenotype. (Circ Res. 2010;107:898-902.)

Key Words: endothelial dysfunction, hypertension, stretch, gene expression, zyxin

Hypertension is a major predisposing factor for endothelial dysfunction,1 causing vascular remodeling and atherosclerosis. However, the mechanism(s) by which a prolonged increase in blood pressure, and thus stretch, translates into a change in endothelial cell (EC) phenotype remains elusive.

Fluid shear stress, the viscous drag of the flowing blood, is the second hemodynamic force sensed by ECs and generally considered to be antiatherosclerotic. In contrast to straight arterial segments, at bifurcations (cyclic) stretch dominates over shear stress, and it is mainly here where endothelial dysfunction and concomitantly atherosclerosis develops. Although there is some insight into shear stress–induced gene expression,2,3 rather little is known about stretch-induced signaling,4 with no systematic analysis of endothelial cell pathways affected by stretch-induced alterations in gene expression available to date. Here, we have comprehensively explored such transcriptional changes in human cultured ECs and in addition have identified the focal adhesion protein zyxin to be critically involved therein.

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.
colocalizes with the nuclear protein Ku80. Given that down-regulation of zyxin affects stretch-induced gene expression in rat vascular smooth muscle cells, we used a siRNA-based approach in the human cultured ECs that resulted in a robust (80%) decrease in zyxin abundance (Online Figure I). Testing of 3 genes known to be stretch-sensitive revealed that the chemokines interleukin (IL)-8 and CXCL1 but not the B-type endothelin receptor (ETB-R) are controlled by zyxin (Figure 2A). Moreover, IL-8 expression, for example, was dependent on both the frequency and magnitude of stretch (Online Figure II) and, in addition to venous ECs, both stretch-induced nuclear translocation of zyxin and gene expression also occur in arterial ECs (Online Figures III and IV).

To get the full picture of these stretch-induced changes in gene expression and the role of zyxin therein, whole genome arrays were performed with 3 individual batches of primary ECs. Apart from comparing quiescent and stretched control

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**Non-standard Abbreviations and Acronyms**

- ChIP: chromatin immunoprecipitation
- EC: endothelial cell
- EMSA: electrophoretic mobility-shift assay
- ET-1: endothelin-1
- GO: Gene Ontology
- GSEA: Gene Set Enrichment Analysis
- IL: interleukin
- KEGG: Kyoto Encyclopedia of Genes and Genomes
- Nu: nucleus
- ODN: oligodeoxynucleotide
- PyPu box: pyrimidine-purine box (zyxin-binding DNA motif)
- siRNA: short interfering RNA

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**Figure 1. Zyxin and stretch-induced nuclear translocation.** A, Representative confocal immunofluorescence images of zyxin relocation in static and stretched (0% to 10% elongation at 0.5 Hz for 6 hours) ECs. Top, Zyxin (Cy3/ red) and the focal adhesion protein paxillin (Cy2/green) are stained. Nuclei (Nu) are counterstained with DAPI (4',6'-diamidin-2-phenylindol). Paxillin colocalizes with zyxin exclusively in focal adhesions (FA; yellow) but not in stress fibers (SF) or in the nucleus (after stretch). Bottom, Partial stretch-dependent colocalization of the nuclear protein Ku80 (Cy2) with zyxin (Cy3). Whereas nuclei in static cells are virtually devoid of zyxin, significant colocalization occurs in response to stretch (insets with magnified exemplary nuclei). B, Western blot analysis of nuclear translocation of zyxin. Nuclei and the cytosol of static and stretched (6 and 12 hours) ECs were subjected to Western blot analysis. Left, Statistical summary (*P<0.05 vs static control, n=5). Right, Exemplary Western blot. Again, Ku80 and paxillin were used as reference proteins.
ECs also cells without or with zyxin knockdown were analyzed side by side. Only gene products not affected by the transfection itself were included in the analysis. Both, pathway analysis (GSEA) based on KEGG and GO (Online Table II) and analyses of single genes revealed a prominent stretch-sensitive genes controlled by zyxin (Online Figure V) but not in stretch-sensitive genes, revealing that zyxin in fact interacts with the promoter region of these genes. Genes that are expressed independently of zyxin did not emerge in this assay (Figure 3A). Moreover, zyxin seemed to be involved in the regulation of rather distinct pathways such as suppression of apoptosis or chemokine release (Online Table II).

Mechanism of Zyxin-Induced Gene Expression

Zyxin appears to associate with the promoter of the human thrombomodulin gene at a stretch of pyrimidines (PyPu box; position −453 to −481; M. Cattaruzza, unpublished observation, 2005). On analyzing the promoter regions of genes identified to be stretch-sensitive, comparable motifs close to the transcription start site were found solely in the 13 stretch-sensitive genes controlled by zyxin (Online Figure V) but not in the 11 zyxin-independent genes. To test whether zyxin indeed associates with this motif, ChIP assays were performed for 9 stretch-sensitive genes, revealing that zyxin in fact interacts with the promoter region of these genes. Genes that are expressed independently of zyxin did not emerge in this assay (Figure 3A).

To corroborate this finding, the cultured ECs were exposed to a decoy ODN mimicking the PyPu box in the human prepro-endothelin-1 (ET-1) gene (edn1; 5’-GCACCTCC-TTCCTTTTCCCGAAG-3’; position −163 to −136). Unlike the scrambled control ODN, the decoy ODN virtually abolished the stretch-induced expression of ET-1, as well as that of prepro-ET-1, another well-known stretch-sensitive gene (Figure 3B). EMSA using a probe with a sequence identical to that of the decoy ODN finally confirmed that in human cultured ECs exposed to cyclic stretch a nuclear protein–DNA complex forms that according to supershift analysis contains zyxin (Figure 3C).

Discussion

Although stretch-induced gene expression in human cultured ECs has been analyzed by DNA microarrays before, the present data to our knowledge provide the first comprehensive pathway analysis. We further demonstrate that in ECs the cytoskeletal protein zyxin acts as a transducer of stretch into the...
nucleus, where it orchestrates the expression of a large subset of stretch-sensitive genes through a novel DNA-response element.

Prolonged hypertension, ie, a long-term increase in circumferential stretch, promotes endothelial dysfunction, which is characterized by an aberrant expression of proinflammatory genes. Our findings substantiate the assumption that ECs respond to such a supraphysiological increase in stretch with the activation of pathways known to be pivotal for the development of atherosclerosis.9,10

In addition, we show that zyxin, a protein normally associated with focal adhesions and stress fibers, plays a major role in stretch-induced endothelial gene expression. Apart from potential effects on the structural and functional integrity of the EC on its loss, zyxin directly interacts with the promoter region of most stretch-sensitive genes. Its binding motif does not resemble a typical consensus sequence but rather a particular base composition, ie, a stretch of pyrimidines. A preliminary "manual" screen of several stretch-sensitive genes revealed that it is only present in the proximal promoter regions of zyxin-regulated genes. This, however, does not exclude the possibility that similar elements may stochastically exist in unrelated regions of the genome as frequently is the case for the binding motifs of conventional transcription factors.

Almost all zyxin-dependent genes can be attributed to a set of well-defined pathways that, on the one hand, inhibit apoptosis and proliferation and strengthen cell–matrix interactions but, on the other hand, contribute to a broad range of proinflammatory responses (Online Figure VI). Such an orchestrated change in the endothelial transcriptome suggests a role for zyxin in vascular remodeling processes, especially when considering that ECs largely govern the phenotype of vascular smooth muscle cells11 and regulate leukocyte migration into the vessel wall.12,13 Although potentially preventing EC damage, this signaling pathway may thus facilitate endothelial dysfunction and consequently both hypertension-induced arterial remodeling and atherogenesis. Future studies must reveal whether the prevention of zyxin-mediated gene expression in ECs in fact constitutes a meaningful therapeutic strategy to combat these vascular complications.

Acknowledgments
We thank Dr Mary Beckerle for providing the zyxin antibodies. We also thank Danijela Heide and Renate Cattaruzza for excellent technical assistance.

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Disclosures
None.

References

Novelty and Significance

What Is Known?
- Supraphysiological cyclic stretch causes a pathological phenotype shift in endothelial cells (ECs).
- This phenotypic alteration, referred to as endothelial dysfunction, is critically implicated in the onset and progression of hypertension-induced arterial remodeling and atherosclerosis.

What New Information Does This Article Contribute?
- The cytoskeletal protein zyxin is a pivotal mediator of stretch-induced endothelial signaling.
- Zyxin also acts as a transcription factor orchestrating the expression of up to 67% of all stretch-induced genes.

Exposure of ECs to an inadequate increase in blood pressure, and hence stretch, provokes a phenotype shift referred to as endothelial dysfunction, which is a key step in the development of atherosclerosis and related vascular remodeling processes. Despite its clinical impact, neither the primary stretch-induced signaling pathways nor the resulting changes in the endothelial transcriptome have as yet been analyzed in detail. Here, we provide first experimental evidence that the cytoskeletal protein zyxin mediates the bulk of stretch-induced endothelial gene expression, acting both as a mechanotransducer and transcription factor. Because zyxin activation mainly accounts for proinflammatory gene expression, the selective blockade of its activity may provide a promising therapeutic concept to target atherosclerosis and related vascular complications.
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Zyxin Mediation of Stretch-Induced Gene Expression in Human Endothelial Cells: Correction

In the article that appears on page 898 of the October 1, 2010, issue, Figures 1 and 2 were not reproduced in color as had originally been intended.
The authors regret this error. This error has been noted and corrected in the online version of the article, which is available at http://circres.ahajournals.org/cgi/content/full/107/7/898

Reference

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Supplement for *Zyxin mediation of stretch-induced gene expression in human endothelial cells* by Agnieszka Wójtowicz, Sahana Suresh, Li Li, Norbert Gretz, Markus Hecker and Marco Cattaruzza

Experimental procedures  
Supplemental Table I  
Supplemental Table IIA to IIC  
Legend to Supplemental Figures I-VI  
References
Experimental procedures

Isolation and growth of endothelial cells. Endothelial cells (ECs) were isolated from umbilical cord veins as described previously (1) and directly cultured on BioFlex™ collagen I elastomers (Flexercell Inc., Hillsborough, NC, USA) using endothelial cell growth medium (Promocell, Heidelberg, Germany). Upon reaching confluence (typically 4 days post isolation), the cells were exposed to cyclic stretch (elongation 10% at 0.5 Hz with a sinusoidal profile), a commonly accepted method to mimic an increase in circumferential wall tension, in a Flexercell FX-3000 strain unit as previously described (2). Umbilical cords were collected after obtaining informed consent from the parents. All procedures were approved by the local ethics committee. For exemplary experiments, human aortic and coronary artery endothelial cells were purchased (PromoCell, Heidelberg, Germany) and cultured in endothelial cell growth medium without hydrocortisone (PromoCell).

Transfection of siRNA. ECs were transfected with short interfering RNA directed against zyxin (siRNA; Hs_ZYX_1_HP) or the AllStars Negative Control (both from Qiagen, Hilden, Germany) by way of magnet-assisted transfection (IBA, Göttingen, Germany). Briefly, for each well of a 6-well plate, 3 µg of siRNA were diluted in Opti-MEM I (Invitrogen, Karlsruhe, Germany) to give a final volume of 200 µl. The transfection complex was formed by adding 3 µl of MATra-si reagent (IBA) to the diluted siRNA followed by mixing and incubation at ambient temperature for 20 minutes. After washing the cells once in Opti-MEM I, 200 µl/well transfection mixture were layered drop wise together with 2 mL Opti-MEM I onto the cells. Cells were then incubated on a custom-made magnetic plate (IBA) which was specifically designed to fit the wells of the BioFlex plate for 15 minutes at 37°C and 5% CO₂. To induce rapid zyxin turnover, cells were briefly exposed to cyclic stretch (30 minutes, 0.5 Hz, 10% elongation) followed by a 72-hour resting period in endothelial cell growth medium which had been determined to be optimal for transfection efficiency.

Decoy oligodeoxynucleotide blockade of zyxin activity. In some experiments, activity of nuclear zyxin was blocked by a specific double-stranded decoy oligodeoxynucleotide (ODN, IBA, Göttingen, Germany). The sequence chosen among several similar motifs was from the
human endothelin-1 gene (edn1, pos. -136 to -163; cf. Figure 1B and supplementary Table I). Additionally, a scrambled ODN with the same base content was used as a control (supplementary Table I).

To effectively prevent the binding of zyxin to the promoter of its target genes, cells were pre-incubated with the decoy ODN for 3 hours prior to their exposure to cyclic stretch at a final concentration of 10 μmol/L. The decoy ODN readily enters cultured endothelial cells without any auxiliary means. In the cell, it effectively blocks target gene expression by mimicking the promoter binding site for, e.g. activating transcription factors (3).

**Electrophoretic mobility shift analysis (EMSA).** Preparation of nuclear extracts and subsequent EMSA were performed as described previously (4), except for the addition of the phosphatase inhibitors sodium fluoride (20 mmol/L) and sodium vanadate (1 mmol/L) to all buffers used. The custom-made 32P-labeled single-stranded ODN had exactly the same sequence as the forward or reversed strand of the decoy ODN (suppplementary Table I) and was hybridized by standard procedures (4). For supershift analyses, two zyxin-reactive rabbit antisera (B38 and B71; kind gift of Dr. Mary Beckerle, Huntsman Center, University of Utah, Salt Lake City, USA,5) was employed. For this purpose, the procedure was exactly as described for the EMSA except for a 30 minute pre-incubation of the nuclear extract with 1 μL antiserum at ambient temperature before incubating the sample with the radioactively labeled ODN.

**Analysis of gene and protein expression.** RNA isolation (RNeasy Kit, Qiagen), reverse transcription of RNA and real time RT-PCR analysis (LC system 1.2, Roche, Mannheim, Germany; for primers see supplementaryTable I) were done as described previously (1,6). The annealing temperature for all primers was 58°C, elongation was for 30 seconds. GAPDH expression was used as an internal standard for normalization. Absolute measures of mRNA are expressed as copy number per ng of total RNA and or as percent of untreated control, respectively.

Western blot analysis was performed according to standard procedures with 7-15 μg protein loaded per lane using a Mini Protean minigel chamber and a Mini Trans-blot cell (Biorad, Munich, Germany). A PVDF blotting membrane and the ECL detection solution from Amersham Pharmacia Biotech (Freiburg, Germany) were used for visualisation of the immunoreactive proteins. As primary antibodies, a polyclonal rabbit anti-zyxin antibody
(B72; dilution 1:1000), a rabbit anti-PARP-1 antiserum (dilution 1:1000; Abcam, United Kingdom) and a monoclonal mouse anti-β-actin antibody (1:5000 each; Sigma-Aldrich, Deisenhofen, Germany) to control for equal loading and transfer were used. Secondary antibodies used were horseradish peroxidase-coupled monoclonal anti-rabbit and anti-mouse IgGs derived from goat (Sigma-Aldrich).

**Immunofluorescence analysis.** For analysis of zyxin localization, cells grown on BioFlex membranes were fixed (methanol/acetone 1:1 for 20 minutes at -20°C). The fixed cells were permeabilized with 0.1% Triton X-100/PBS (phosphate-buffered saline: 8.0 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4, 0.24 g KH2PO4 per L, pH = 7.6) and nonspecific binding was blocked using casein serum (0.25 % casein, 0.1 % BSA, 15 mM NaN3, 50mM Tris pH 7.6). The membranes were then incubated cell side down with 15 μL of primary antibodies diluted in blocking buffer for 2 hours at room temperature (zyxin rabbit antiserum B72/1:250; paxillin monoclonal mouse antibody from BD Transduction laboratories, Germany/1:100; Ku80 monoclonal mouse antibody from Novus Biologicals, Cambridge, United Kingdom/1:400; CD31 monoclonal mouse, Dako, Glostrup, Denmark/1:25; COX-1 monoclonal mouse antibody from Cayman chemical, Ann Arbor, USA/1:100 and NOS-3 monoclonal mouse antibody from BD Transduction laboratories/ 1:100). After 5 wash steps (5 minutes each, PBS), cells, depending on the primary antibodies used, were incubated cell side down with Cy2- and/or Cy3-conjugated secondary antibodies (1 hour, room temperature, donkey-derived anti-mouse and anti-rabbit antibodies; Jackson Laboratories via Dianova, Hamburg, Germany). The cells were then washed 2 times with PBS followed by a nuclear stain with DAPI (1 μg/mL; 10 min at room temperature) and mounted on to a large coverslip with ProLong (Invitrogen, Oregon, USA).

For confocal microscopy analysis a IX81 microscope equipped with a IX-DSU disk unit and the MT20 multi-wavelength illumination system was used in combination with the cell^R software package (Olympus, Hamburg, Germany).

**Chromatin immunoprecipitation (ChIP).** ChIP assays were performed by using the ChIP-IT Express Kit (Active Motif; Rixensart, Belgium) according to the manufacturer’s instructions (all buffers and enzymes not defined were provided by the manufacturer). In brief, ECs were
exposed to cyclic stretch (10% 0.5 Hz for 6 h) as described above. Thereafter, cells were fixed and cross-linked with formaldehyde (1% in PBS), the DNA was isolated and digested with the provided restriction enzyme cocktail and subjected to immunoprecipitation. The antibodies used for this procedure were from Dr. Mary Beckerle (5, zyxin: rabbit antiserum B71; the antiserum B72 used for Western blot and other procedures did not yield any reproducible results), Sigma-Aldrich (mouse β-actin as a negative control) and Active Motif (RNA polymerase II/ChIP-certified). The resulting precipitated DNA was subjected to gene-specific PCR (see supplementary Table I and Figs. 3 and online Fig. VI) and the amount of amplified DNA was densitometrically analysed after agarose gel electrophoresis (Molecular Imager Gel Doc XR System and the Density One densitometry software version 4.6; Biorad). The optimal number of PCR cycles (95°C/30 s denaturation, 60°C/30 s annealing and 72°C/1 minute synthesis) varied between 28 (clusterin after RNA polymerase II precipitation) and 37 (most genes after precipitation with B71). The primers were chosen in a way that putatively functional PyPu boxes were included in the amplified regions.

DNA microarray analysis. A total of 12 independent microarray analyses was performed to (i) characterize all stretch-dependent gene products in the endothelial cells and (ii) to analyze which subset of genes is regulated by zyxin. To this end, 3 individual preparations of ECs were used to compare untreated cells under static conditions to untreated cells exposed to cyclic stretch for 6 hours (10% elongation, 0.5 Hz, see above). Another 3 individual cell preparations were used to analyze stretch-stimulated gene expression in ECs after siRNA-mediated knockdown of zyxin (cf. Figure I and above) or transfection with control siRNA to account for potentially unspecific effects of the transfection procedure itself (not shown). RNA was isolated as described above and reverse-transcribed to cDNA using Superscript II (Invitrogen). This was followed by in vitro transcription using the GeneChip IVT Labeling Kit (Affymetrix, High Wycombe, UK) to obtain biotin-labeled RNA. Hybridization and detection of the labeled RNA on the Affymetrix HG-U133 Plus 2.0 human whole genome chip were performed according to the instructions of the manufacturer. Microarray data were submitted to NCBI GEO (7) at http://www.ncbi.nlm.nih.gov/geo/query; sample number [GSE17814]. Differential gene expression was analyzed based on a log linear mixed model
ANOVA using the JMP Genomics software package, version 3.2 (SAS Institute, Cary, NC, USA). Custom CDF (8) with Unigene based gene/transcript definitions (version 11) was used to annotate the arrays. Signals were first log transformed and quantile normalized, before differential gene expression was revealed by ANOVA.

Pathway analysis. Gene Set Enrichment Analysis 2.0 (GSEA 2.0; 9) was applied to reveal biological pathways regulated by cyclic stretch and/or zyxin. Genes were ranked according to their expression changes. Gene Ontology terms related to molecular function (GO_mf) and KEGG pathways were examined using 1000 rounds of permutation of gene sets as described in reference 9.

Statistical analysis. All quantitative data are presented as means ± SEM of n observations with cells/samples obtained from individual umbilical cords. Unpaired Student’s t-test or repeated-measure ANOVA followed by a Tukey-Kramer post-hoc test were performed as appropriate by using the InStat software package version 3.06 (GraphPad Software, San Diego, USA) with a P-value < 0.05 considered to be significant.
Supp. Table 1. Oligonucleotides used for RT-PCR analysis and as decoy ODN. Fragment size/position and GenBank accession numbers are given in the right column. The symbol ‘**’ depicts phosphorothioate-modifications which were introduced into decoy ODN for added intracellular stability.

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<th>Gene product (ChIP)</th>
<th>Sequence</th>
<th>Acc. number position (size)</th>
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<tr>
<td>CLU (ChIP)</td>
<td>ACCAAACGTGGATCTGCAAG GTTGTTGGGC ACTGGGAGG</td>
<td>NC_000008.10 81/-632 (713 bp)</td>
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<tr>
<td>CXCL1</td>
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<td>ET-1 (ChIP)</td>
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<td>NC_000006.11 19/-202 (221 bp)</td>
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<td>ETB-R</td>
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<td>NM_001122659.1 70/171 (102 bp)</td>
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<td>GAPDH (real time)</td>
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<td>GAPDH (conventional)</td>
<td>TCACCATCTTCCAGGAGCG CTGCTTACACCACCTTCTTG</td>
<td>BC083511 273/844 (582 bp)</td>
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<td>Gucy1B3 (ChIP)</td>
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<td>GATGTGCTAGTCCACCACACCATCCTG</td>
<td>NM_031935.2 4568/4683 (115 bp)</td>
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<tr>
<td>Hemicentin (ChIP)</td>
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<td>HEY-1</td>
<td>TGAGAGCGAATGGAGGAGC AAGTTACCTTCCCTCCTTCG</td>
<td>NM_012258.3 440/550 (111 bp)</td>
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               | CAGTAGGAGGCGAGTGATAG | NC_000005.9  
               | -657/-957 (301 bp) |
| ICAM-1 (ChIP) | TGATGGGGCAGTCAACAGCTA  
                | GGGTAAGGTTCTTGGCCCCTA | NM_000201.2  
                | 610/716 (107 bp) |
| ICAM-1 (ChIP) | GTGCATGAGGCGCTGGGTTC  
                | GGCCTCCTCTCTCTACAC | NC_000019.9  
                | -699/-1043 (345 bp) |
| IL-8 (ChIP)  | TAGCCAGGATCCACAAGTCC  
                | GCTTCCACATGTCTACA | NM_000584.2  
                | 879/995 (117 bp) |
| IL-8 (ChIP)  | CACTCCATCCCTTTTGTCTAG  
                | CTTAAGAAGGTTGGGTCTATC | NC_000004.11  
                | -764/-1201 (438 bp) |
| JAM2 (ChIP)  | ATCCGGATCAAAAAATGTGAC  
                | GCTGGAGCCACTAATACTTCC | NM_021219.2  
                | 825/949 (125 bp) |
| JAM-2 (ChIP) | CTCAGCTTCCGGCCGTGGGC  
                | CTCTGAGGAGGTGCAGGGGTCC | NC_000021.8  
                | -24/-623 (600 bp) |
| Laminin-γ    | ATCTGATGGACACAGCCTCTC  
                | CCTCTCTTCAGCTTGACA | NM_005562.2  
                | 3634/3758 (125 bp) |
| E-Selectin   | CGCCCATCCCTCAGCCTCAGA  
                | GGCCCTCGCAACGTGAAACT | NM_011345.2  
                | 1054/1170 (117 bp) |
| VCAM-1 (ChIP) | CATGGAATTCCGAACCCAAACA  
                  | GACCAAGACGGTTGGATCTCAGG | NM_001078.2  
                  | 1593/1674 (82 bp) |
| VCAM-1 (ChIP) | GATTCCAGACCTCAGCTATG  
                  | GTATCCAGCTCTGAAGCC | NC_000001.10  
                  | -77/-1430 (1506 bp) |
| Zyxin        | CTGTCCCTCAGCTGGATG  
                  | GAGTTGGACCTGAGGCTTG | NM_003461.4  
                  | 609/867 (259 bp) |
| decoy ODN    | A*C*C*AGGCACCTCCTCCTCCC*G*A*A | NC_000006.11 (pos. -163 to -136) |
| control ODN  | T*C*C*GCACATGCCATTACTGTTCAC*C*C*T | N.A. |
Supp. Tables IIA-C: Selective GSEA-pathway analysis of stretch-sensitive genes in human endothelial cells (third column) and the role of zyxin therein (fourth column). All pathways are derived from the canonical pathway sets provided by KEGG (http://www.genome.jp/kegg/) and GO (pathways molecular function; http://www.geneontology.org). Only pathways significantly altered ($p \leq 0.02$) by cyclic stretch and/or zyxin knock down have been included; significant $p$-values are printed bold. As an estimate for the actual grade of enrichment and activation/inhibition of a given pathway, normalized enrichment scores* (NES) are listed. Additionally, false discovery rates** (FDR) have been included; a value of FDR $\leq 0.4$ is regarded to be significant. Of a total of 598 pathways analyzed, 43 were characterized to be stretch-sensitive. Of those, 29 could be shown to be zyxin-dependent approximately reflecting the situation at the single gene level. For analyses with altered parameters please use the raw data provided at NCBI GEO (http://www.ncbi.nlm.nih.gov/geo/query) using the sample number [GSE17814]. (a) Stretch-regulated zyxin-independent pathways. (B) Stretch-regulated zyxin-induced/-maintained pathways. (C) Stretch-induced zyxin-repressed pathways.

* In GSEA all genes are ranked with respect to the degree of changes in expression. The normalized enrichment score NES reflects the degree to which a pathway (defined by a set of genes) is overrepresented at the top or bottom of this ranked list of genes for a given comparison (e.g., quiescent ECs vs. stretched ECs). Thus, a very high value for NES (e.g., NES > 1.8) reflects a marked activation of the pathway. Accordingly, a value of, e.g., NES < -1.6 defines a significant inhibition of the pathway.

** The false discovery rate FDR provides a help to estimate the probability that a pathway defined to be significantly regulated by a high NES is falsely positive. E.g., FDR = 0.3 defines the probability that 7 of 10 predictions by GSEA are correct.

***Several other regulated cancer pathways have been excluded as these somewhat redundantly focus on signaling pathways included elsewhere in this table.
Supplemental Table IIA: Stretch-regulated zyxin-independent pathways.

<table>
<thead>
<tr>
<th>No.</th>
<th>Pathway (Source)</th>
<th>Functional Description</th>
<th>stretch vs. quiescent p (NES/FDR)</th>
<th>stretch vs. quiescent/ Zyxin knock down p (NES/FDR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GTPASE_ACTIVATOR_ACTIVITY (GO pathway_mf)</td>
<td>0.008 (1.58/0.35)</td>
<td>0.012 (1.73/0.42)</td>
<td></td>
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<tr>
<td>2</td>
<td>CALMODULIN_BINDING (GO pathway_mf)</td>
<td>0.005 (1.68/0.48)</td>
<td>0.005 (1.77/0.58)</td>
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<tr>
<td>3</td>
<td>BIOSYNTHESIS_OF_STEROIDS (KEGG pathway)</td>
<td>0.0000 (-2.11/0.00)</td>
<td>0.009 (-1.79/0.05)</td>
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<tr>
<td>4</td>
<td>MAPK_SIGNALING_PATHWAY (KEGG pathway)</td>
<td>0.012 (1.39/0.30)</td>
<td>0.019 (1.31/0.40)</td>
<td></td>
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<tr>
<td>5</td>
<td>CELL_ADHESION_MOLECULES (KEGG pathway)</td>
<td>0.02 (-1.34/0.36)</td>
<td>0.0000 (-1.79/0.04)</td>
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</tr>
<tr>
<td>6</td>
<td>REGULATION_OF_ACTIN_CYTOSKELETON (KEGG pathway)</td>
<td>0.003 (1.32/0.38)</td>
<td>0.060 (1.22/0.47)</td>
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</tr>
<tr>
<td>7</td>
<td>GLYCAN_STRUCTURES_DEGRADATION (KEGG pathway)</td>
<td>0.014 (-1.62/0.19)</td>
<td>0.002 (-1.86/0.037)</td>
<td></td>
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<tr>
<td>8</td>
<td>GLUTAMATE_METABOLISM (KEGG pathway)</td>
<td>0.002 (1.75/0.06)</td>
<td>0.002 (1.76/0.11)</td>
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<tr>
<td>9</td>
<td>PHOSPHORUS_OXYGEN_LYASE_ACTIVITY (GO pathway_mf)</td>
<td>0.0000 (-1.85/0.114)</td>
<td>0.036 (-1.51/0.51)</td>
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<tr>
<td>10</td>
<td>BLADDER_CANCER (KEGG pathway)***</td>
<td>0.0000 (1.96/0.0076)</td>
<td>0.005 (1.68/0.18)</td>
<td></td>
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<tr>
<td>11</td>
<td>CYCLASE_ACTIVITY (GO pathway_mf)</td>
<td>0.0000 (-1.89/0.108)</td>
<td>0.084 (-1.44/0.56)</td>
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<td>12</td>
<td>ENDONUCLEASE_ACTIVITY_GO_0016893 (GO pathway_mf)</td>
<td>0.037 (1.55/0.33)</td>
<td>0.0000 (1.89/0.37)</td>
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<tr>
<td>13</td>
<td>ANTIGEN_PROCESSING_AND_PRESENTATION (KEGG pathway)</td>
<td>0.059 (-1.41/0.40)</td>
<td>0.0018 (-1.99/0.017)</td>
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<tr>
<td>14</td>
<td>HEMATOPOIETIC_CELL_LINEAGE (KEGG pathway)</td>
<td>0.0024 (1.61/0.16)</td>
<td>0.026 (1.45/0.32)</td>
<td></td>
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**Supplemental Table IIB:** Stretch-regulated zyxin-induced/-maintained pathways.

<table>
<thead>
<tr>
<th>No.</th>
<th>Pathway (Source) Functional Description</th>
<th>stretch vs. quiescent p (NES/FDR)</th>
<th>stretch vs. quiescent/ Zyxin knock down p (NES/FDR)</th>
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<tbody>
<tr>
<td>15</td>
<td>CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION (KEGG pathway)</td>
<td><strong>0.0000</strong> (1.80/0.06)</td>
<td><strong>0.001</strong> (-1.5/0.17)</td>
</tr>
<tr>
<td>16</td>
<td>PROTEIN_KINASE_ACTIVITY (GO pathway_mf)</td>
<td><strong>0.0000</strong> (1.43/0.30)</td>
<td>0.502 (-0.98/0.85)</td>
</tr>
<tr>
<td>17</td>
<td>FOCAL_ADHESION (KEGG pathway)</td>
<td><strong>0.0000</strong> (1.59/0.17)</td>
<td>0.054 (1.27/0.47)</td>
</tr>
<tr>
<td>18</td>
<td>AMINO_ACID_TRANSMEMBRANE_TRANSPORTER_ACTIVITY (GO pathway_mf)</td>
<td><strong>0.004</strong> (1.77/0.72)</td>
<td>0.164 (1.24/0.62)</td>
</tr>
<tr>
<td>19</td>
<td>POLYMERASE_II_TRANSCRIPTION_FACTOR_ACTIVITY (GO pathway_mf)</td>
<td><strong>0.018</strong> (1.40/0.40)</td>
<td>0.767 (-0.85/0.91)</td>
</tr>
<tr>
<td>20</td>
<td>TRANSCRIPTION_COREPRESSOR_ACTIVITY (GO pathway_mf)</td>
<td><strong>0.017</strong> (1.44/0.44)</td>
<td>0.337 (-1.07/0.76)</td>
</tr>
<tr>
<td>21</td>
<td>CHEMOKINE_RECEPTOR_BINDING (GO pathway_mf)</td>
<td>0.046 (1.46/0.45)</td>
<td><strong>0.0000</strong> (-2.30/0.0)</td>
</tr>
<tr>
<td>22</td>
<td>CHEMOKINE_ACTIVITY (GO pathway_mf)</td>
<td>0.048 (1.46/0.46)</td>
<td><strong>0.0000</strong> (-2.40/0.0)</td>
</tr>
<tr>
<td>23</td>
<td>CYTOKINE_ACTIVITY (GO pathway_mf)</td>
<td>0.057 (1.30/0.46)</td>
<td><strong>0.0000</strong> (-1.96/0.02)</td>
</tr>
<tr>
<td>24</td>
<td>COMPLEMENT_AND_COAGULATION_CASCADES (KEGG pathway)</td>
<td>0.475 (1.00/0.77)</td>
<td><strong>0.0049</strong> (-1.70/0.07)</td>
</tr>
<tr>
<td>25</td>
<td>TOLLLIKE_RECEPTOR_SIGNALING_PATHWAY (KEGG pathway)</td>
<td>0.439 (1.00/0.78)</td>
<td><strong>0.0000</strong> (-1.90/0.04)</td>
</tr>
<tr>
<td>26</td>
<td>TGF_BETA_SIGNALING_PATHWAY (KEGG pathway)</td>
<td>0.246 (1.14/0.72)</td>
<td><strong>0.0000</strong> (-1.82/0.04)</td>
</tr>
<tr>
<td>27</td>
<td>G_PROTEIN_COUpled_RECEPTOR_BINDING (GO pathway_mf)</td>
<td>0.086 (1.35/0.42)</td>
<td><strong>0.0000</strong> (-2.21/0.00)</td>
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<td>28</td>
<td>HYDROLASE_ACTIVITY_HYDROLYZING_O GLY COSYL_COMPOUNDS (GO)</td>
<td>0.638 (-0.87/0.98)</td>
<td><strong>0.0000</strong> (-1.89/0.04)</td>
</tr>
</tbody>
</table>
**Supplemental Table IIC:** Stretch-induced zyxin-repressed pathways.

<table>
<thead>
<tr>
<th>No.</th>
<th>Pathway (Source) Functional Description</th>
<th>stretch vs. quiescent $p$ (NES/FDR)</th>
<th>stretch vs. quiescent/Zyxin knock down $p$ (NES/FDR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>LIPID_TRANSPORTER_ACTIVITY (GO pathway_mf)</td>
<td>0.277 (-1.15/0.74)</td>
<td><strong>0.009</strong> (-1.79/0.12)</td>
</tr>
<tr>
<td>30</td>
<td>DORSO_VENTRAL_AXISFORMATION (KEGG pathway)</td>
<td><strong>0.0000</strong> (2.05/0.00)</td>
<td>0.718 (0.82/0.90)</td>
</tr>
<tr>
<td>31</td>
<td>OXIDOREDUCTASE_ACTIVITY (GO pathway_mf)</td>
<td><strong>0.0000</strong> (-1.52/0.37)</td>
<td>0.434 (-1.01/0.78)</td>
</tr>
<tr>
<td>32</td>
<td>P53_SIGNALING_PATHWAY (KEGG pathway)</td>
<td>0.090 (-1.18/0.31)</td>
<td><strong>0.0000</strong> (1.76/0.13)</td>
</tr>
<tr>
<td>33</td>
<td>CELL_CYCLE (KEGG pathway)</td>
<td><strong>0.008</strong> (-1.57/0.18)</td>
<td><strong>0.0000</strong> (2.05/0.02)</td>
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<tr>
<td>34</td>
<td>DNA_POLYMERASE (KEGG pathway)</td>
<td><strong>0.008</strong> (-1.62/0.17)</td>
<td>0.111 (1.33/0.40)</td>
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<tr>
<td>35</td>
<td>PYRIMIDINE_METABOLISM (KEGG pathway)</td>
<td>0.186 (-1.18/0.60)</td>
<td><strong>0.005</strong> (1.48/0.31)</td>
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<tr>
<td>36</td>
<td>STRUCTURAL_CONSTITUENT_OF_CYTOSKELETON (GO pathway_mf)</td>
<td><strong>0.004</strong> (-1.59/0.28)</td>
<td>0.956 (-0.64/0.97)</td>
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<tr>
<td>37</td>
<td>GAP_JUNCTION (KEGG pathway)</td>
<td>0.117 (-1.26/0.54)</td>
<td><strong>0.009</strong> (1.48/0.30)</td>
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<tr>
<td>38</td>
<td>LYASE_ACTIVITY (GO pathway_mf)</td>
<td><strong>0.003</strong> (-1.67/0.30)</td>
<td>0.706 (-0.85/0.91)</td>
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<td>39</td>
<td>VALINE_LEUCINE_AND_ISOOLEUCINE_DEGRADATION (KEGG pathway)</td>
<td><strong>0.0000</strong> (-1.99/0.01)</td>
<td>0.16 (-1.25/0.45)</td>
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<tr>
<td>40</td>
<td>GLUTATHIONE_METABOLISM (KEGG pathway)</td>
<td>0.41 (1.03/0.78)</td>
<td><strong>0.005</strong> (1.60/0.19)</td>
</tr>
<tr>
<td>41</td>
<td>ASCORBATE_AND_ALDARATE_METABOLISM (KEGG pathway)</td>
<td>0.701 (0.81/0.91)</td>
<td><strong>0.006</strong> (1.66/0.17)</td>
</tr>
<tr>
<td>42</td>
<td>PATHOGENIC_ESCHERICHIA_COLI_INFECTION_EHEC (KEGG pathway)</td>
<td>0.045 (1.45/0.26)</td>
<td><strong>0.0000</strong> (2.00/0.02)</td>
</tr>
<tr>
<td>43</td>
<td>PATHOGENIC_ESCHERICHIA_COLI_INFECTION_EPEC (KEGG pathway)</td>
<td>0.082 (1.39/0.30)</td>
<td><strong>0.0000</strong> (2.00/0.01)</td>
</tr>
</tbody>
</table>
Legends to supplemental figures:

**Supp. Figure I: siRNA-mediated knock down of zyxin.** Western blot analysis of zyxin expression in ECs pre-treated with scrambled control or zyxin-specific siRNA. \*$_p < 0.05$ vs. untreated control; $n = 6$. The insert depicts an exemplary Western blot.
Supp. Figure II: Analysis of the impact of the stretch protocol on stretch-induced IL-8 expression. (A) Real time-PCR analysis of static and stretch-induced (10%, 6 hours) IL-8 expression at 0.2 to 2 Hz. *p < 0.05 vs. static control, n = 3-10. (B) Real time-PCR analysis of static and stretch-induced (0.5 Hz, 6 hours) IL-8 expression at 3 to 20% stretch. Higher stretch levels (>25%) resulted in disruption of the cells (not shown). *p < 0.05 vs. static; n = 3-10.
Supp. Fig. II Wojtowicz et al.
Supp. Figure III: Immunofluorescence analysis of coronary and aortic cultured endothelial cells: (A) Representative confocal immunofluorescence images of human cultured aortic and coronary ECs. Typical endothelial cell gene products (NOS-3 (I), COX-1 (II) and zyxin/CD31 (III)) are expressed and localized in a typical way. (B) Immunofluorescence analysis of zyxin relocation in static and stretched (0-10%, 0.5 Hz, 6 hours) aortic (upper panel) and coronary artery (lower panel) ECs. Zyxin (Cy3/red) and the focal adhesion protein paxillin (Cy2/green) were stained. Nuclei are counter-stained with DAPI. Paxillin is co-localized with zyxin exclusively in the focal adhesions (yellow) but not in stress fibers or in the nucleus (after stretch).
Supp. Figure IV: Analysis of the impact of the endothelial cell phenotype on stretch-induced IL-8 expression: Real time-PCR analysis of static and stretch-induced (10%, 0.5 Hz, times as indicated) IL-8 expression in coronary artery (left panel) and aortic ECs (right panel). *p < 0.05 vs. static control, n = 3-6.
Supp. Fig. IV Wojtowicz et al.
**Supp. Figure V: Zyxin-DNA interactions:** (A) Alignment of stretch-response element-like sequences found in the subset of zyxin-dependent genes: hairy/enhancer-of-split related with YRPW motif 1 (Hey1), interleukin-8 (IL-8), hemicentin-1 (HMCN1), E74-like factor 4 (ELF4), prepro-endothelin-1 (ET-1), laminin C3 (LAMC3), the transcription factor forkhead box O1 (FOXO1), notch homolog-2 (Notch2), HMG-CoA reductase (HMGCR), E-selectin (SEL-E), ICAM-1, VCAM-1, integrin-β6 (ITGB6). Stretch-inducible zyxin-independent genes which have been unsuccessfully analyzed for this motif, code for matrix metalloproteases-1 and 12 (MMP-1/12), von Willebrand factor (vWF), clusterin (CLU), guanylate cyclase-1β (GUCY1B3), frizzled-like-8 (FZD8), heme oxygenase-1 (HMOX1), CD34, the transcription factor forkhead box C1 (FOXC1), leptin receptor (LEPR), matrilin-2 (MATN2), prominin-1 (PROM1) and sulfatase-1 (SULF1). The position relative to the first ATG codon is given in parentheses. (B) Exemplary pictures of ChIP analyses for all gene products tested for stretch-induced gene expression (10% elongation, 0.5 Hz, 6 hours) and the involvement of zyxin therein. Besides the zyxin-dependent genes IL-8, VCAM-1, HMNC, Hey-1, HMGCR and ICAM-1, zyxin-independent gene products (GUCY, JAM2 and CLU) and ET-1 are shown.
**Zyxin-activated genes**

ELF4 (-266): 5' - CTCCCTCGG CTCTTCCCTC CCTCCCGA - 3'
ET-1 (-136): 5’ - GCCAGSCGCT TCCTTTCTCTC CCCGTAAG - 3’
HMCN1 (-670): 5’ - GCGTTTCCTC CCGTCTTCCTT CCTGCAGA - 3’
IL-8 (-685): 5’ - GTCTTACAT TCTTTCTCTC TCTGATAG - 3’
LAMC2 (-76): 5’ - CCGGGAGCCC TCCCTCTCTC CCTCCGGGTG - 3’
FOXO1 (-597): 5’ - AGATTCTCTT TCTCCTTCTC AGAGGTTC - 3’
Notch2 (-446): 5’ - ATGTGAAATC CTCCCTCTTC TGAGCTGA - 3’
HMGCR (-475): 5’ - AGGCTGCCG CTCCTCCCTC TTCTCTCTCTCCGGCTG - 3’

**Zyxin-repressed genes**

SEL-E (-230): 5’ - CTAACACCTG TCTTTTCTCTT TTGACCTG - 3’
ICAM-1 (-473): 5’ - TCACGCAGCT TCCTTTCTCTT TTCTGGA - 3’
VCAM-1 (-315): 5’ - TGGCTCCATC TTTTTCTCTC CCACCC - 3’
ITGB6 (-606): 5’ - CTCTCTCTCTC TCTTTCTCTC CATTACT - 3’
Hey-1 (-228): 5’ - CCGCGCCTCC TCCCTCCCTC GAGTGCAG - 3’

The motif was not found in these stretch-induced but zyxin-independent genes: CD34, Clusterin, FOXC1, GUCY1B3, JAM2, LEPR, MATN2, MMP-1/12, PROM1, SULF1 & vWF

**B**

<table>
<thead>
<tr>
<th>α-pol 2</th>
<th>α-zyxin</th>
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</thead>
<tbody>
<tr>
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<td>&lt;clusterin</td>
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<tr>
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<td>&lt;ET-1</td>
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<tr>
<td>&lt;GUCY-1B3</td>
<td>&lt;GUCY-1B3</td>
</tr>
<tr>
<td>&lt;Hey-1</td>
<td>&lt;Hey-1</td>
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<tr>
<td>&lt;HMGCR</td>
<td>&lt;HMGCR</td>
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<tr>
<td>&lt;HMNC1</td>
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<tr>
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<tr>
<td>&lt;VCAM-1</td>
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</table>

- + stretch
Supp. Figure VI: Simplified scheme of the consequences of zyxin-mediated changes in endothelial cell gene expression: Comparison of phenotypic changes in stretch-induced (0-10% elongation at 0.5 Hz for 6 hours) endothelial cell gene expression in the absence or presence of zyxin (for details refer to the expanded methods section and to supplemental Table II above).
Supp. Fig. VI Wojtowicz et al.

Wall tension (cyclic stretch) → zyxin

- Matrix stabilization
- MAP kinase signalling ↑
- Cell-cell adhesion ↓
- Stabilized cytoskeleton

zymxin

Chemokine signalling
- Cytokine signalling
- Complement activity
- Coagulation

Cell-matrix contacts ↑
- Trimeric G-protein signalling ↑
- p53-signalling ↓ (anti-apoptotic)
- Proliferation ↓
References


