iScience, Volume *23*

Supplemental Information

Quantitative Proteomics Identifies TCF1 as a Negative Regulator of *Foxp3* Expression in Conventional T Cells

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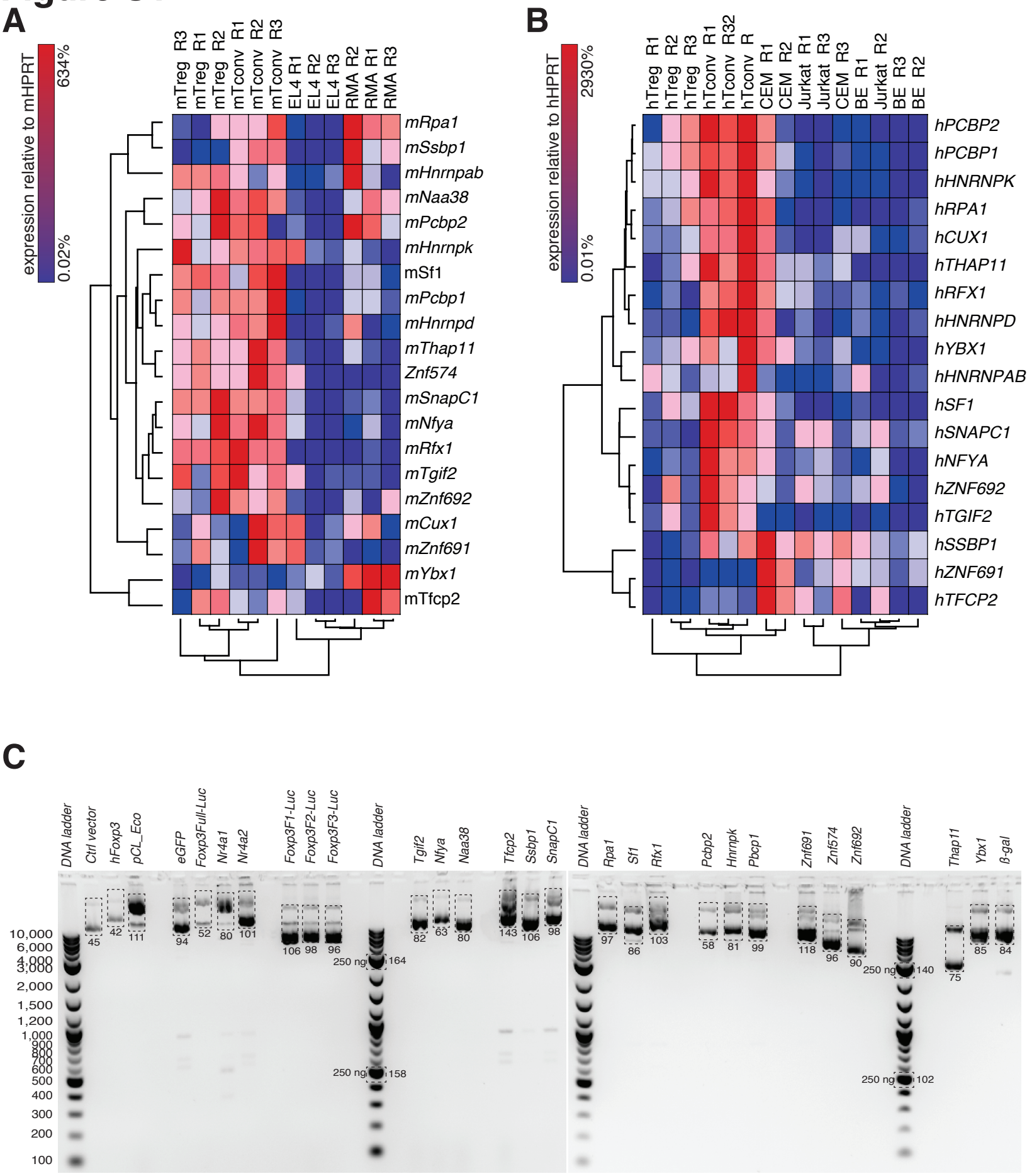
Figure S1


A
expression relative to mHPRT
634%
0.02%
mTreg R3
mTreg R1
mTreg R2
mTconv R1
mTconv R2
mTconv R3
EL4 R1
EL4 R2
EL4 R3
RMA R2
RMA R1
RMA R3
mRpa1
mSsbp1
mHnrnpab
mNaa38
mPcbp2
mHnrnpk
mSf1
mPcbp1
mHnrnpd
mThap11
Znf574
mSnapC1
mNfya
mRfx1
mTgif2
mZnf692
mCux1
mZnf691
mYbx1
mTfcp2
B
expression relative to hHPRT
2930%
0.01%
hTreg R1
hTreg R2
hTreg R3
hTconv R1
hTconv R32
hTconv R
CEM R1
CEM R2
Jurkat R1
Jurkat R3
CEM R3
BE R1
Jurkat R2
BE R3
BE R2
hPCBP2
hPCBP1
hHNRNPK
hRPA1
hCUX1
hTHAP11
hRFX1
hHNRNPD
hYBX1
hHNRNPAB
hSF1
hSNAPC1
hNFYA
hZNF692
hTGIF2
hSSBP1
hZNF691
hTFCP2

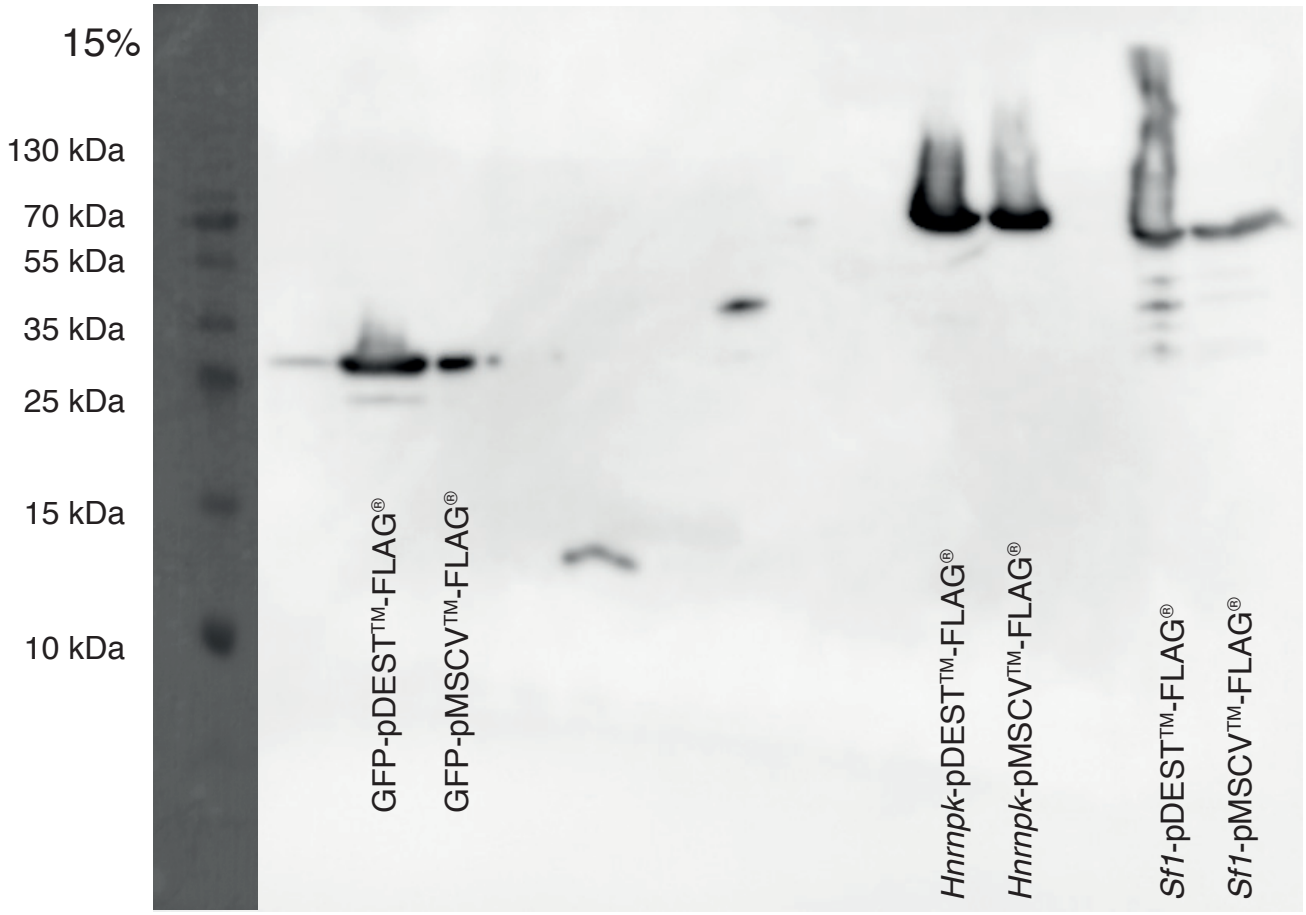
C

DNA ladder
Ctrl vector
hFoxp3
pCL_Eco
eGFP
Foxp3Full-Luc
Nr4a1
Nr4a2
Foxp3F1-Luc
Foxp3F2-Luc
Foxp3F3-Luc
DNA ladder
Tgif2
Nfya
Naa38
Tfcp2
Ssbp1
SnapC1
DNA ladder
Rpa1
Sf1
Rfx1
Pcbp2
Hnrnpk
Pbcp1
Znf691
Znf574
Znf692
DNA ladder
Thap11
Ybx1
β-gal
10,000
6,000
4,000
3,000
2,000
1,500
1,200
1,000
900
800
700
600
500
400
300
200
100
45
42
111
94
52
80
101
106
98
96
250 ng
164
250 ng
158
82
63
80
143
106
98
97
86
103
58
81
99
118
96
90
250 ng
140
250 ng
102
75
85
84

Figure S1. Gene expression and plasmid stability, related to Figure 2. (**A**) Gene expression of candidate transcription factors in primary murine T_{reg} or T_{conv} cells or cell lines. Rows: different genes; Columns: different replicates of samples; heatmap row-normalized, n=3. (**B**) Gene expression of candidate transcription factors in primary human T_{reg} or T_{conv} cells or cell lines. Rows: different genes; Columns: different replicates of samples; heatmap row-normalized, n=3. (**C**) Plasmid stability and size analysis: different vectors (Luciferase vectors, eukaryotic production vectors, viral production vectors) were linearized and separated on agarose gel to visualize plasmid integrity. Numbers indicate concentration by measuring dark values in rectangular section.

Figure S2

A



B

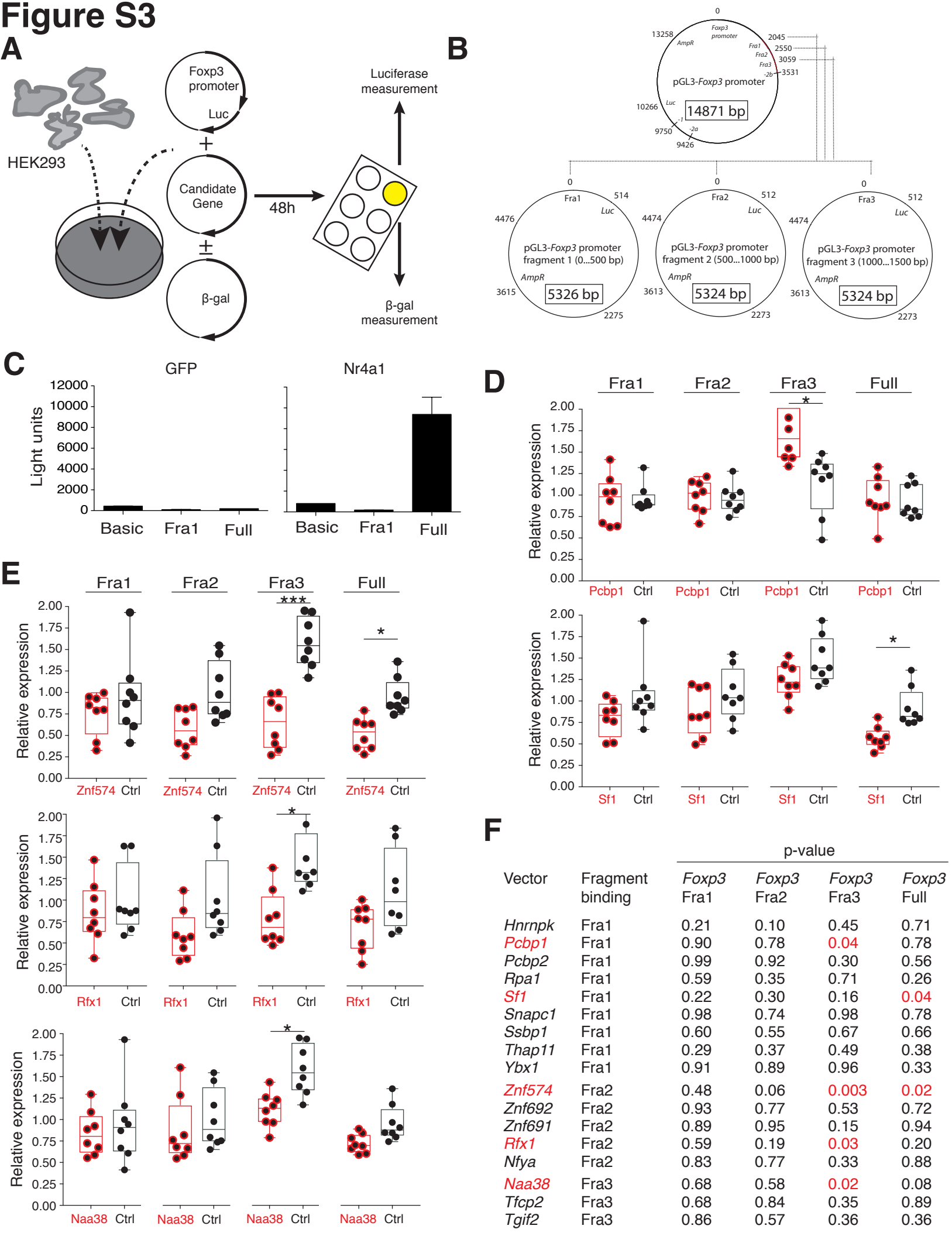
Gene expression (%Gapdh)

	Transfected pDEST plasmid																	
Plasmid		*Hnrnpk*	*Rpa1*	*Naa38*	*Tgif2*	*Znf574*	*Thap11*	*Ybx1*	*Pcbp2*	*Pcbp1*	*Ssbp1*	*Nfya*	*Snapc1*	*Znf691*	*Rfx1*	*Sf1*	*Znf692*	*Tfcp2*
Plasmid ID	H2O	252	248	244	242	255	257	258	251	253	246	243	247	254	250	249	256	245
mHnrnpk	0,00	**85,40**	0,20	0,20	0,20	0,30	0,20	0,40	1,20	0,90	0,00	0,00	0,00	0,40	0,30	1,00	1,40	2,80
mRpa1	0,00	0,00	**174,50**	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
mNaa38	0,00	0,00	0,00	**1043,20**	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
mTgif2	0,00	0,00	0,00	0,00	**177,20**	0,00	0,00	0,00	0,00	0,00	0,01	0,08	0,00	0,01	0,01	0,02	0,05	0,08
mZnf574	0,00	0,00	0,00	0,00	0,00	**8,60**	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
mThap11	0,00	0,10	0,10	0,10	0,00	0,10	**97,70**	0,10	0,00	0,10	0,11	0,09	0,00	0,10	0,06	0,07	0,06	0,07
mYbx1	0,00	18,20	22,60	24,90	29,10	21,50	32,40	**478,30**	45,50	46,80	35,65	44,75	34,92	42,43	23,83	44,78	33,17	40,05
mPcbp2	0,00	6,20	5,80	7,20	6,70	5,10	6,00	9,30	**208,60**	7,40	8,62	7,41	7,39	8,26	4,26	8,15	4,91	5,39
mPcbp1	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	**1008,40**	0,01	0,01	0,01	0,01	0,00	0,01	0,01	0,02
mSsbp1	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,10	0,10	0,10	**28,50**	0,01	0,01	0,01	0,01	0,01	0,01	0,02
mNfya	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,10	0,10	0,10	0,04	**201,67**	0,04	0,07	0,03	0,06	0,04	0,05
mSnapc1	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	**281,28**	0,00	0,00	0,00	0,00	0,00
mZnf691	0,00	0,00	0,00	0,00	0,00	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,00	**672,72**	0,00	0,00	0,00	0,00
mRfx1	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	**42,69**	0,00	0,00	0,00	**0,64**	0,00	0,00	0,00
mSf1	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,01	0,03	0,01	0,00	**153,58**	0,01	0,00
mZnf692	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	**42,37**	0,00
mTfcp2	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	**356,77**

Top 1 or top 2 item (s) highlighted in red and bold

Figure S2. Transgene expression and plasmid identity, related to Figure 2. (**A**) Western Blot with lysates of producer cell lines transfected with either pDEST eukaryotic production vectors or pMSCV viral production vectors. Recombinant proteins were FLAG-labeled and detected with anti-FLAG antibodies. Protein ladder to the left. (**B**) Eukaryotic production vectors were transfected into production cell lines. Afterwards, cells were lysed and RNA was isolated. cDNA was generated and gene expression was measured by qPCR. Rows = different qPCR primers; Columns: different eukaryotic production vectors used to transfect target cells.

Figure S3



Vector	Fragment binding	p-value *Foxp3* Fra1	*Foxp3* Fra2	*Foxp3* Fra3	*Foxp3* Full
Hnrnpk	Fra1	0.21	0.10	0.45	0.71
Pcbp1	Fra1	0.90	0.78	0.04	0.78
Pcbp2	Fra1	0.99	0.92	0.30	0.56
Rpa1	Fra1	0.59	0.35	0.71	0.26
Sf1	Fra1	0.22	0.30	0.16	0.04
Snapc1	Fra1	0.98	0.74	0.98	0.78
Ssbp1	Fra1	0.60	0.55	0.67	0.66
Thap11	Fra1	0.29	0.37	0.49	0.38
Ybx1	Fra1	0.91	0.89	0.96	0.33
Znf574	Fra2	0.48	0.06	0.003	0.02
Znf692	Fra2	0.93	0.77	0.53	0.72
Znf691	Fra2	0.89	0.95	0.15	0.94
Rfx1	Fra2	0.59	0.19	0.03	0.20
Nfya	Fra2	0.83	0.77	0.33	0.88
Naa38	Fra3	0.68	0.58	0.02	0.08
Tfcp2	Fra3	0.68	0.84	0.35	0.89
Tgif2	Fra3	0.86	0.57	0.36	0.36

Figure S3. Testing of candidate factors with *Foxp3*-Luciferase vectors in HEK293 cells, related to Figure 2. (**A**) Experiment overview: 50,000 HEK293 cells were seeded in a 96-well flat-bottom plate and transfected with 3 vectors 24h later: β-galactosidase normalization vector, *Foxp3*-Luciferase reporter vector, and a eukaryotic expression vector containing the transgene of interest. After 48 h, cells were lysed and β-galactosidase as well as luciferase enzymatic activity was measured on a luminometer with automated injection of substrates. (**B**) Top: Overview of Foxp3-promoter vector with Promega pGL3 Luciferase reporter. Below: constructs with small *Foxp3* promoter sequences (500 bp) were cloned from the larger construct. (**C**) Transfection of HEK293 cells with *GFP*-transgene (left) or *Nr4a1* transgene (right) with a basic Luciferase reporter vector (Basic), a *Foxp3*-Fra1 reporter vector (Fra1) or the full *Foxp3* promoter vector (Full). X-axis vector, y-axis light units (Luciferase). (**D**) Co-transfection of HEK293 cells with either *Pcbp1*, *Sf1* or control (*GFP*) transgene, a *Foxp3* Luciferase vector and β-galactosidase normalization vector. X-axis indicates transgene and vector type, y-axis relative expression normalized to basic vector background. Statistical analysis with unpaired t-test (n=8, *p<0.05). (**E**) Co-transfection of HEK293 cells with either *Znf574*, *Rfx1*, *Naa38* or control (*GFP*) transgene, a *Foxp3* Luciferase vector and β-galactosidase normalization vector. X-axis indicates transgene and vector type, y-axis relative expression normalized to basic vector background. Statistical analysis with unpaired t-test (n=8, *p<0.05 and ***p<0.001). (**F**) Transfection of HEK293 cells with all transgenes using the assay described in (A). Transgenes were co-transfected with either the basic vector, *Foxp3*-Fra1, *Foxp3*-Fra2, *Foxp3*-Fra3 or a full *Foxp3* promoter vector as explained above. In the table, p-value is reported (unpaired t test, n=8). Significant values are highlighted in red.

Figure S4

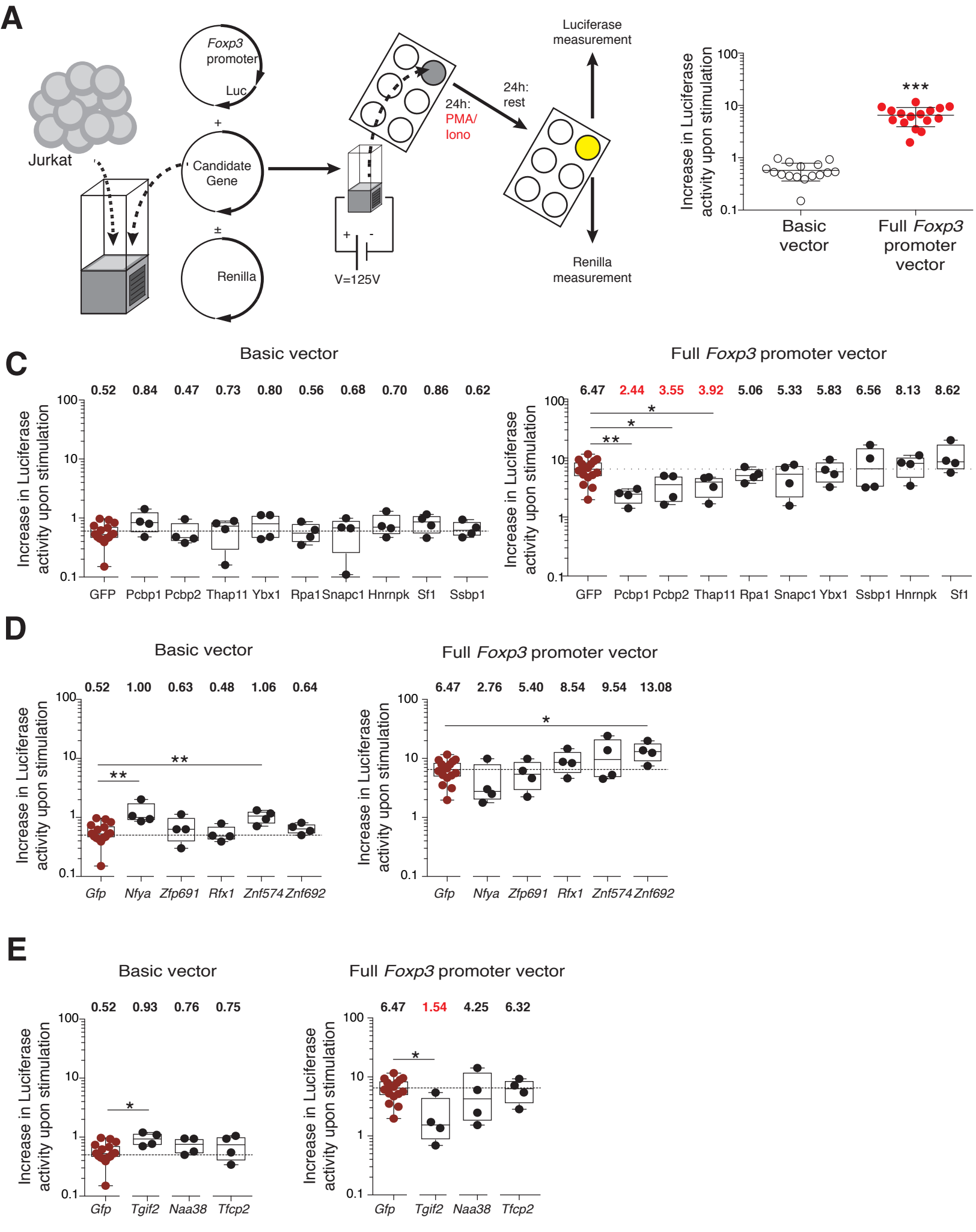

A
Jurkat
Foxp3 promoter
Luc
+
Candidate Gene
±
Renilla
+
-
V=125V
24h: PMA/ Iono
24h: rest
Luciferase measurement
Renilla measurement
B
Increase in Luciferase activity upon stimulation
100
10
1
0.1
***
Basic vector
Full Foxp3 promoter vector
C
Basic vector
Full Foxp3 promoter vector
Increase in Luciferase activity upon stimulation
0.52 0.84 0.47 0.73 0.80 0.56 0.68 0.70 0.86 0.62
GFP Pcbp1 Pcbp2 Thap11 Ybx1 Rpa1 Snapc1 Hnrnpk Sf1 Ssbp1
6.47 2.44 3.55 3.92 5.06 5.33 5.83 6.56 8.13 8.62
*
*
**
GFP Pcbp1 Pcbp2 Thap11 Rpa1 Snapc1 Ybx1 Ssbp1 Hnrnpk Sf1
D
Basic vector
Full Foxp3 promoter vector
Increase in Luciferase activity upon stimulation
0.52 1.00 0.63 0.48 1.06 0.64
**
**
Gfp Nfya Zfp691 Rfx1 Znf574 Znf692
6.47 2.76 5.40 8.54 9.54 13.08
*
Gfp Nfya Zfp691 Rfx1 Znf574 Znf692
E
Basic vector
Full Foxp3 promoter vector
Increase in Luciferase activity upon stimulation
0.52 0.93 0.76 0.75
*
Gfp Tgif2 Naa38 Tfcp2
6.47 1.54 4.25 6.32
*
Gfp Tgif2 Naa38 Tfcp2

Figure S4. Testing of candidate factors with *Foxp3*-Luciferase vectors in Jurkat cells, related to Figure 2 and Figure 3. (**A**) Experiment overview as in **Figure 3D**: 2,000,000 Jurkat cells were electroporated with a Renilla normalization vector, *Foxp3*-Luciferase reporter vector, and a eukaryotic expression vector containing the transgene of interest. 24h after electroporation, cells were stimulated with PMA/Ionomycin. 20h after stimulation, cells were lysed and Renilla as well as Luciferase enzyme activity was measured on a luminometer with automated injection of substrates. (**B**) Jurkat cells were electroporated with the basic vector containing no promoter in front of the Luciferase reporter and with the full *Foxp3* promoter vector. Luciferase reads were Renilla-normalized to adapt for electroporation efficiency changes. Then, normalized values were compared between unstimulated and stimulated conditions to calculate the increase in Luciferase activity upon stimulation (y-axis). Statistical analysis based on unpaired t test (n=16, ***p<0.001). (**C-E**) Jurkat cells were electroporated with either the full *Foxp3* promoter vector or the basic vector, with a Renilla normalization vector and the transgene vector of interest, as described above. Left, increase in Luciferase values upon stimulation with the basic vector, right with the full *Foxp3* promoter vector. Statistical evaluation based on unpaired t testing (n= 4-16, *p<0.05 and **p<0.01). Data are derived from four or more independent experiments.

Gene name	Direction	Sequence
Mouse Cux1	Forward	TGACCTGAGCGGTCCTTACA
Mouse Cux1	Reverse	TGGGGCCATGCCATTTACATC
Mouse Hnrnpab	Forward	ATGGCGGCTACGACTACTC
Mouse Hnrnpab	Reverse	GCTGGCTCTTTCCGTAATTTGT
Mouse Hnrnpd	Forward	GTGAAGTTGTAGACTGCACTCTG
Mouse Hnrnpd	Reverse	CCAAAACCCCTTGATCGCC
Mouse Hnrnpk	Forward	CAGCTCCCGCTCGAATCTG
Mouse Hnrnpk	Reverse	ACCCTATCAGGTTTTCCTCCAA
Mouse Naa38	Forward	GGCTGTTATTACTTCTGATGGCA
Mouse Naa38	Reverse	ACACCACTTGTTCTACTCCCT
Mouse Nfya	Forward	GTTAATGGTGCAAGTCAGTGGA
Mouse Nfya	Reverse	TCTGCTGTAAACCTTGTGTTCC
Mouse Pcbp1	Forward	GACGCCGGTGTGACTGAAA
Mouse Pcbp1	Reverse	GTCAGCGTGATGATCCTCTCC
Mouse Pcbp2	Forward	GCCAGATTTGACCAAGCTGC
Mouse Pcbp2	Reverse	GAGCTGGATTCAATGCCACTG
Mouse Rfx1	Forward	GTTCACGTTGCTCAAGAGGTA
Mouse Rfx1	Reverse	TACTGGTAGGTGCTAGAGCGG
Mouse Rfx1	Forward	AGTACCCGGAGACGCCTATC
Mouse Rfx1	Reverse	CTGCCGGACACATACATGG
Mouse Rpa1	Forward	ACATCCGTCCCATTTCTACAGG
Mouse Rpa1	Reverse	CTCCCTCGACCAGGGTGTT
Mouse Sf1	Forward	AGCCGATGGAACCAAGACAC
Mouse Sf1	Reverse	GCACTATGTAAGCTCTTTCCTGT
Mouse Sf1	Forward	AGAAGACCTGACTCGTAAACTGC
Mouse Sf1	Reverse	CCCTCGCTGTTGTAGATTGGT
Mouse Snapc1	Forward	CGCTTCCAAGAGATGGACAG
Mouse Snapc1	Reverse	CGTGTGTGGAGGCAAAAAGTAG
Mouse Ssbp1	Forward	CAACAAATGAGATGTGGCGATCA
Mouse Ssbp1	Reverse	ACGAGCTTCTTACCAGCTATGA
Mouse Tfcp2	Forward	TGAGTGATGTCCTCGCATTGC
Mouse Tfcp2	Reverse	TCGTTCTCATTATCGGGAGGC
Mouse Tgif2	Forward	ATGTCGGACAGCGATCTAGG
Mouse Tgif2	Reverse	TCCCGGAGGATCTTTACTGAC
Mouse Thap11	Forward	ATGCCTGGCTTTACGTGCT
Mouse Thap11	Reverse	GGTGGGTTGGAAGGTGGAG
Mouse Ybx1	Forward	CAGACCGTAACCATTATAGACGC
Mouse Ybx1	Reverse	ATCCCTCGTTCTTTTCCCCAC
Mouse Zfp574	Forward	ACATTGAGCACCGCTATGTCT
Mouse Zfp574	Reverse	CTCTCTTGGATGAGGGTCTGATA
Mouse Zfp691	Forward	GGAGAAGGGGCTAAACCTTGG
Mouse Zfp691	Reverse	GCAGTGACTTTCTGCCTTGTCT
Mouse Zfp691	Forward	GGAGAGTGGATGGCTCAAAGG
Mouse Zfp691	Reverse	CGTTCTCAGGTTGGAGGTATTGT
Mouse Zfp691	Forward	ATACCTCCAACCTGAGAACGC
Mouse Zfp691	Reverse	GGCGCATTGGTAGTGCTTC
Mouse Zfp692	Forward	GGTGCTCCTGTCTCACACAC
Mouse Zfp692	Reverse	CTGCTTAGGTACATCTGAAGGTG

Gene name	Direction	Sequence
Human Cux1	Forward	GAAGAACCAAGCCGAAACCAT
Human Cux1	Reverse	AGGCTCTGAACCTTATGCTCA
Human Foxp3	Forward	GTGGCCCGGATGTGAGAAG
Human Foxp3	Reverse	GGAGCCCTTGTCGGATGATG
Human Hnrnpab	Forward	ACCGAGAACGGACATGAGG
Human Hnrnpab	Reverse	GCCACCAACGAACATTTTTCC
Human Hnrnpd	Forward	GCGTGGGTTCTGCTTTATTACC
Human Hnrnpd	Reverse	TTGCTGATATTGTTCCTTCGACA
Human Hnrnpk	Forward	CAATGGTGAATTTGGTAAACGCC
Human Hnrnpk	Reverse	GTAGTCTGTACGGAGAGCCTTA
Human Hnrnpk	Forward	GCAGGAGGAATTATTGGGGTC
Human Hnrnpk	Reverse	TGCACTCTACAACCCTATCGG
Human Naa38	Forward	GCATTCGCATGACAGATGGAC
Human Naa38	Reverse	CGACGGCTTGAGGAACTCC
Human Nfya	Forward	CAGTGGAGGCCAGCTAATCAC
Human Nfya	Reverse	CCAGGTGGGACCAACTGTATT
Human Nfya	Forward	TGAAGGGCAGACCATCGTCTA
Human Nfya	Reverse	TCCTGTTTGAACAATCTGTGCT
Human Pcbp1	Forward	GCCGGTGTGACTGAAAGTG
Human Pcbp1	Reverse	CCCAATGATGCTTCCTACTTCC
Human Pcbp1	Forward	AAGAAAGGGGAGTCGGTTAAGA
Human Pcbp1	Reverse	GCCGGTCAGAGTGATGATTCTC
Human Pcbp2	Forward	ACTCTCACCATCCGGCTACTT
Human Pcbp2	Reverse	TCGCGCATCTTCTTAACTGATTC
Human Pcbp2	Forward	GCGCAGATCAAAATTGCGAAC
Human Pcbp2	Reverse	ATATTGAGCCAGGCTAATGCTG
Human Rfx1	Forward	CGTGGCTCAAGAGGTGCAG
Human Rfx1	Reverse	TCTCGGGATAGGAGTAGGTGC
Human Rfx1	Forward	CGGCAAGCACCAGCTACTAC
Human Rfx1	Reverse	GGACACGTACATGGGCATGG
Human Rpa1	Forward	GGGGATACAAACATAAAGCCCA
Human Rpa1	Reverse	CGATAACGCGGCGGACTATT
Human Rpa1	Forward	CGGGAATGGGTTCTACTGTTTC
Human Rpa1	Reverse	CGAGCACAAATGGTCCACTTG
Human Sf1	Forward	GAAGACCTGACTCGTAAACTGC
Human Sf1	Reverse	CCTCGCTATTGTAGATGGGCT
Human Sf1	Forward	GGAGCGGCACAACCTCATC
Human Sf1	Reverse	CCGGATCATAATCTTGGCATTGC
Human Snapc1	Forward	CGGACAGTGTACGCTTCGAG
Human Snapc1	Reverse	ATCGCCAAGCCAAAGCTAAAG
Human Snapc1	Forward	AGAGTTGGTGCTTTGTATCTGC
Human Snapc1	Reverse	GCTCTGTCTAGTCGTAGCTTCC
Human Ssbp1	Forward	TGAGTCCGAAACAACTACCAGT
Human Ssbp1	Reverse	CCTGATCGCCACATCTCATTAG
Human Ssbp1	Forward	ACTGGGTGATGTCAGTCAAAAG
Human Ssbp1	Reverse	TGCTTGTCGCCTCACATTATT
Human Tfcp2	Forward	TCTGGCCGACGAAGTGATTG
Human Tfcp2	Reverse	ATCAGGAGGCAAACTCGACTC
Human Tfcp2	Forward	GTGTTCCATGACAGAAGGCTT
Human Tfcp2	Reverse	TTATACCCACAGACATCGGGAT
Human Tgif2	Forward	TGACCCCTGGTAGCACACTTA
Human Tgif2	Reverse	GTGGTGGCGTGTTGAAGAGT

Human Thap11	Forward	ATGCCTGGCTTTACGTGCT
Human Thap11	Reverse	GCGTCCTTTGGAAACGTGTAG
Human Thap11	Forward	ATACTGGCTCCGACCATTCG
Human Thap11	Reverse	CTTGGCCTCAGTGAGACGC
Human Ybx1	Forward	GGGGACAAGAAGGTCATCGC
Human Ybx1	Reverse	CGAAGGTACTTCCTGGGGTTA
Human Ybx1	Forward	CCCCAGGAAGTACCTTCGC
Human Ybx1	Reverse	AGCGTCTATAATGGTTACGGTCT
Human Zfp574	Forward	ACATTGAGCACCGCTATGTCT
Human Zfp574	Reverse	CCTGCACAAGGGTCTGATAGA
Human Zfp574	Forward	AGACCCTTGTGCAGGAGAG
Human Zfp574	Reverse	GTGGTGCCTTAGGTGATGGC
Human Zfp691	Forward	GAGCAGAGTCCAGAACCACAC
Human Zfp691	Reverse	GCAGTTCATCCGACAGGCT
Human Zfp691	Forward	TCGGATGAACTGCAAGAAACTC
Human Zfp691	Reverse	TGTGTTCTCAGGTTGGAGGTA
Human Zfp692	Forward	TTCCGCACTAGCAGCAACC
Human Zfp692	Reverse	AAACCCGCATATCTCACACTG
Human Zfp692	Forward	TGTGAGATATGCGGGTTTACCT
Human Zfp692	Reverse	TGACTCTTGAGGGGCTAGAAG

Table S1. Sequences of qPCR primers for mouse and human genes, related to Figure 2.

TRANSPARENT METHODS

Ethics statement

Peripheral blood mononuclear cells for CD4 T-cell enrichment were isolated from leukocyte reduction chambers. Collection of blood cells from healthy donors was performed in compliance with the Helsinki Declaration. All donors signed an informed consent. The leukapheresis procedure and subsequent purification of immune cells were approved by the local ethical committee (reference number 13-101-0240).

Mice / cell lines

Animals were housed under specific pathogen-free conditions at the RCI or the DKFZ, and the governmental committee for animal experimentation (Regierungspräsidium Karlsruhe, Germany for DKFZ Heidelberg or Regierungspräsidium Unterfranken, Würzburg for Regensburg) approved all animal experiments. All experiments were conducted in accordance with the relevant regulatory standards. In these experiments, we used both female and male adult (>8 weeks of age) wild type C57/BL6 mice or Foxp3$^{GFP,\ DTR,\ CD45.1}$ C57/BL6 mice (Kim et al., 2007). TCF1-deficient animals were a received from Hans Clevers (Tcf7^{tm1Cle,} (Castrop et al., 1995; Verbeek et al., 1995)). For cell lines, we used a human embryonic kidney cell line (ATCC® CRL-1573™ and ATCC® CRL-3214™), a murine EL4 T-cell line (ATCC® TIB-39™), and a human Jurkat JE6.1 T-cell line (ATCC® TIB-152™). Cells were incubated at standard TC conditions (37°C, 5% CO2) in complete medium and were regularly tested for mycoplasma infection and contamination with other cell types.

Isolation of nuclear protein

Nuclear protein was isolated from EL4 T cells with the NXtract isolation kit (Sigma-Aldrich), and protein concentration was measured using a BCA kit (Thermo-Fisher #23225). Upon isolation of nuclear protein, a selective enrichment of nuclear protein of 5.5-fold compared to the cytosolic fraction as well as enrichment for nucleus-associated proteins based on gene ontology of detected peptides was achieved. About 40 mg of nuclear protein were used for each replicate.

Preparation of *Foxp3* Fra1, Fra2, and Fra3 probes

Short *Foxp3*-promoter fragments were produced from a Full *Foxp3* promoter vector with a biotinylated forward primer and standard reverse primers. PCR conditions for the production of biotinylated PCR primers were optimized to reduce a contamination with unbound biotinylated PCR primers. PCR products were purified using a quick PCR purification kit (Life Technologies #K3100-01). To measure biotinylation of the probe, the PCR product and its individual primers were plotted onto a PVDF membrane and UV cross-linked. Biotinylation was identified with an anti-biotin HRP and chromogenic detection.

Quantitative proteomics with *Foxp3* promoter DNA probes

The procedure is visualized in **Figure 1B** and described in literature (Mittler et al., 2009). First, carefully washed streptavidin beads were linked individually to *Foxp3*-Fra1 probes, *Foxp3*-Fra 2 probes, or *Foxp3*-Fra 3 probes for 3 hours at RT. Then, free bead binding sites were blocked and beads were washed again. Nuclear protein was pre-incubated with unlabeled beads to remove non-specific bead-binding proteins. The cleared nuclear

protein was then added to each probe-labeled bead and incubated on a rotating wheel for 3 hours at 4°C. After incubation, beads were washed to remove unbound protein and bead-bound sequence-specific proteins were eluted. Protein was trypsin-digested and peptides were labeled with stable isotopes by dimethylation of N-termini and lysines. Samples were then combined and reduced by isoelectric focusing. Finally, samples were subjected to nanoLC-MS analysis, which allows the quantitative detection of peptides that were originally bound to specific probe-labeled beads.

Real-time PCR to verify expression levels of candidate proteins

First, we synthesized and tested Sybr primers for both mouse and human candidate proteins with the aid of a public database (http://pga.mgh.harvard.edu/primerbank/). Primer efficiency and melting curve characteristics were analyzed, with efficiencies between 80% and 120% and single melting curves as criteria. We then isolated RNA from cell lines, FACS-sorted primary T_{reg} and T_{conv} cells or plasmid-transfected 293 cells with the RNeasy mini kit. RNA was concentration-adjusted and reversely transcribed using Reverse Transcriptase II and oligo(dT) primers (Life Technologies) according to manufacturer's standards. Real-time PCR was performed with Sybr Master Mix (Applied Biosystems) and Sybr primers. Sybr primer sequences are listed in **Table S1**.

Cloning of candidate genes and evaluation of proper expression for downstream reporter assays

First, we generated PCR products containing intron-free coding DNA for each candidate protein, either from mouse splenic cDNA or commercially available vector clones. PCR products were ligated into pENTR/D-TOPO® vectors (Life Technologies) and sequenced

for proper gene orientation and exclusion of mutations. Once complete, pENTR® vectors were used to shuttle coding DNA into destination vectors such as pDEST26® for eukaryotic overexpression and pMSCV-CD90.1® for viral transduction of T cells with LR clonase II enzyme (Life Technologies). Vectors were sequence-verified to exclude mutations. To exclude vector mix-ups and demonstrate eukaryotic expression, we transfected HEK293 cells with each pDEST® eukaryotic production vector and isolated RNA 48hrs post transfection followed by reverse transcription and real-time PCR. To verify transgene protein expression, we re-shuttled some pENTR®-based genes into FLAG®-tagged pDEST® eukaryotic production vectors and performed Western-Blot based detection of FLAG®-tagged protein with an anti-FLAG® antibody. Size and band intensity were used to identify the transgene of interest. Finally, we validated vector DNA integrity on agarose gels to ensure vector stability and concentration for downstream analyses.

Molecular cloning of short *Foxp3* promoter luciferase vectors

We used a full *Foxp3* promoter luciferase vector (Sekiya et al., 2011) as a template to create short *Foxp3* promoter Fragment 1, Fragment 2 and Fragment 3 PCR products with gene-specific primers including restriction-enzyme binding sites: *Foxp3* Fra 1 (ForP with *Xho*I CTAGCTCGAGACTGCTAGAGGGGGATCAGC and RevP with *Sbf*1 GATCCCTGCAGGGCAGGCTTCAGATCCCTTCT), *Foxp3* Fra 2 (ForP with *Xho*I CTAGCTCGAGCTGCCATGTGAATGGGAAG and RevP with *Sbf*1 GATCCCTGCAGGCCTGGGCCGCTATGTGTAT) and *Foxp3* Fra 3 (ForP with *Xho*I CTAGCTCGAGCCAGGGTCCTAGTCCTGTCA and RevP with *Sbf*1 GATCCCTGCAGGGTTGGCTTCAGGAAAACTGG). The Full *Foxp3* promoter vector was then digested with *Xho*I and *Sbf*1 restriction enzymes to remove the *Foxp3* promoter

sequence, size-separated and isolated from an agarose gel, and treated with phosphatase to prevent re-ligation. Next, the individual small fragments 1,2, or 3 were ligated into the empty vector. The *Foxp3* Fra1, Fra2, and Fra3-pGL3 vectors were sequenced to confirm proper orientation of the small *Foxp3* fragments into the luciferase reporter vector pGL3. All vector sequences and a vector map are supplied in the source data file.

Luciferase-based reporter assays in HEK 293 cells

First, we optimized the dual luciferase reporter system for cell seeding numbers, incubation time, linearity of the luciferase system, and transfection efficiencies. In our optimized protocol, we seeded 50,000 HEK 293 cells into a 96-well flat bottom plate on day 1. After overnight cell attachment, we added 125 ng each of three vectors: first, the β-galactosidase (β-gal) transfection normalization vector; second, a luciferase reporter vector, either the Full, Fra1, Fra2, or Fra3 *Foxp3* promoter vector; third, we added the transgene of interested in a eukaryotic production vector. A total of 375 ng of plasmid DNA were transfected into each well. For transfection, we used the Lipofectamine® transfection system (Life Technologies) according to manufacturer's recommendation. In short, the DNA-water mix containing all three vectors was mixed with a Lipofectamine®-medium suspension and incubated for 5 minutes at RT for liposome formation. Then, the liposomal mix was added to the cell culture and incubated for 24 hours. 50% of medium was exchanged followed by an additional 24 hours of incubation. 48 hours after transfection, cell culture medium was aspirated and cells were lysed with respective lysis buffer from a Dual Light luciferase kit (Thermo Fisher). 75 µL of supernatant were transferred to a black 96-well plate and 12.5 µL of buffer A was added. We then automatically injected 50 µL of

Buffer B plus X-GAL substrate and measured luciferase signals for the pGL3 luciferase vector on a luminometer (Berthold). After sixty minutes, another 50 µL of Accelerator-II solution were injected and the b-galactosidase signal was measured.

Normalization of luciferase values

To normalize transfection efficiency differences, we averaged β-gal light intensity values across all transfected wells of a 96-well plate. We then divided the β-gal readings of each individual well by the average β-gal intensity to determine a relative transfection efficiency reading. The measured luciferase values were then corrected for transfection differences by normalization with the respective β-gal ratio for each individual well.

$$\text{Transfection efficiency A1} = \frac{\text{Individual read (β-gal) A1}}{\text{Average (β-gal) across 96w plate}}$$

$$\text{Normalized luciferase A1} = \frac{\text{Individual read (luciferase) A1}}{\text{Transfection efficiency A1}}$$

Calculation of specific binding

To test for unspecific binding effects to elements on the pGL3 luciferase vector other than the integrated *Foxp3* promoter, we measured all our candidate proteins against a control-pGL3 vector, which does not contain any relevant promoter sequence before the luciferase ORF. We then cross-compared the normalized luciferase values for the Full *Foxp3* promoter vector as well as Fra1, Fra2 and Fra3 *Foxp3* promoter vectors against the control pGL3 vector to determine sequence-specific up-or downregulation of gene expression. To measure whether any of our candidate proteins significantly up-or downregulate *Foxp3* promoter activity, we compared normalized luciferase expression

values between cells co-transfected with GFP, a non-nuclear protein without transcription factor activity, and cells co-transfected with a candidate *Foxp3*-promoter binding protein.

$$\text{Sequence-specific binding} = \frac{\text{Norm. luciferase value for Foxp3-luciferase vector A2}}{\text{Norm. luciferase value for control-luciferase vector A1}}$$

Significance level for protein X = t.test (Specific binding GFP vs. Specific binding X)

Luciferase-based reporter assays in TCR-stimulated Jurkat cells

Analogously to the screenings described above, we used a three-vector system to check the effects of our candidate proteins in Jurkat T cells: The first vector was a luciferase reporter vector containing the Full *Foxp3* promoter sequence (5000 ng per test); the second vector was a eukaryotic production vector carrying the candidate gene (5000 ng per test); third, we used a Renilla-based normalization vector (500 ng per test). Before electroporation, Jurkat T cells were counted and adjusted to $2x10^6$ cells per electroporation. Cells were washed with OptiMEM medium and a mix of all three plasmids was added to each Jurkat cell preparation. Cells were transferred to electroporation cuvettes (Biorad) and electroporated with 125 V of electric current and “Mammalian 11 – Jurkat” settings with a Biorad electroporation machine. Afterwards, cells were transferred into pre-warmed six-well plates with 1500 µL of complete medium. 24 hours after incubation, electroporated cells were either stimulated with PMA (100 ng/µL) and Ionomycin (1000 ng/µL) or left untreated. 20 hours after stimulation, cells were washed and resuspended in 330 µL 1X lysis buffer (Promega) and lysed for 15 minutes at RT. Lysate was transferred to black 96-well plates, with 120 µL of luciferase measurements and 30 µL for Renilla measurements. Luciferase substrate and Renilla substrate were

freshly prepared (details see Appendix) and 100 µL were injected followed by 10s reading time on a luminometer (Berthold).

Calculation of specific binding for the Jurkat T cell screening

Similar to our transfection efficiency calculation for the HEK293-cell based screenings, we first averaged the Renilla transfection control values across all Renilla-transfected and non-stimulated samples. We then divided the individual Renilla read per well by the average reading to yield a measure of electroporation efficiency for each well. It should be noted that we used electroporation efficiencies of unstimulated wells to normalize PMA/Ionomycin-treated samples, since PMA specifically induces activity on the Renilla vector and thereby causes false-positive results also in the luciferase channel.

$$\text{Electroporation efficiency A1} = \frac{\text{Individual read (Renilla) A1}}{\text{Average (Renilla) across experiment}}$$

$$\text{Normalized luciferase A1} = \frac{\text{Individual read (luciferase) A1}}{\text{Electroporation efficiency A1}}$$

Next, we calculated normalized luciferase values for wells carrying the Full *Foxp3* pGL3 luciferase vector plus a selected transgene and calculated the relative induction compared to non-stimulated controls. These values were combined across four independent experiments and used to check for significant down-regulators in comparison to GFP controls.

$$\text{Relative Induction (protein X)} = \frac{\text{Normalized luciferase of stimulated well protein X}}{\text{Normalized luciferase of unstimulated well protein X}}$$

Significance level for protein X = t.test (Rel. Induction GFP vs. Rel. Induction X)

Viral transduction of candidate genes into primary induced T_{reg} cells

Retrovirus in the pMSCV-CD90.1® system can be manufactured in PhxEco cells, a pCL-Eco (packaging plasmid) carrying variant of HEK 293 cells. Therefore, PhxEco cells were seeded on a gelatin matrix at 400,000 cells per well in a six well plate 24 hours before lipofection. To produce liposomal particles containing the viral transgene, we co-incubated 3000ng of vector DNA and 1000ng of additional pCL-Eco packaging plasmid with 12 µL of TransIT-293® transfection reagent (Mirus) for 20 minutes at RT. Liposomes were added to PhxEco-carrying six-well plates and incubated for an additional 16 hours. CD4 T cells were enriched from whole spleen using anti-mouse CD4 biotinylated antibody (Biolegend Clone RM4-5) and anti-biotin ultrapure microbeads (Miltenyi Biotec). Cells were purified using magnetic columns (Miltenyi Biotec). Cells were activated with anti-mouse CD3/28 microbeads (Miltenyi T_{reg} expansion kit mouse #130-095-925) *in-vitro*. We also added 50 ng/mL TGF-β to T-cell cultures to induce Foxp3 expression. Two days after cell seeding, we added viral supernatant carrying pMSCV® retrovirus with the transgenes of interest. T cells were transduced by six hours of incubation at 37°C. Afterwards, viral supernatant was removed and cells were incubated with fresh medium supplemented with IL-2 and TGF-β for another 72 hours. Then, cells were harvested and surface-stained with CD4, CD90.1 and a live/dead exclusion dye, followed by fixation and intracellular staining for Foxp3 protein expression. Cells were analyzed on a BD CantoII™ or LSRII™ flow cytometer, and transduction efficiency was assessed by CD90.1 transgene expression level.

Viral transduction of candidate genes into primary T_{reg} cells

Virus was generated in PhxEco production cells. T_{reg} cells were isolated from murine spleens via bead-based pre-enrichment and FACS-based sorting of $CD4^+CD25^+Foxp3(GFP)^+$ T_{reg} cells on ARIAII™ or ARIAIII™ high-speed cell-sorting systems. T_{reg} cells were supplemented with high-dose IL-2 (2000 U/mL) and anti CD3-CD28 microbeads (Miltenyi T_{reg} expansion kit mouse #130-095-925). 48hrs after stimulation, T_{reg} cells were virally transduced as described above. 72hrs after viral transduction, T_{reg} cells were harvested and surface-stained with CD4, CD90.1 and a live/dead exclusion dye, followed by fixation and intracellular staining for Foxp3 protein expression. Cells were analyzed on a BD CantoII™ or LSRII™ flow cytometer, and transduction efficiency was assessed using CD90.1 transgene expression level.

CRISPR/Cas9-mediated deletion of TCF1 in mouse T cells

CD4 T cells were enriched from whole spleen using anti-mouse CD4 biotinylated antibody (Biolegend Clone RM4-5) and anti-biotin ultrapure microbeads (Miltenyi Biotec). Cells were purified using magnetic columns (Miltenyi Biotec) and FACS. Cells were activated with anti-mouse CD3/28 microbeads (Miltenyi) and IL-2 (Novartis, Proleukin S, 500 units/ml) for 3 days *in-vitro*. To induce Foxp3, TGF-β (Peprotech 100-21) was added at different concentrations. Knock-down was performed with 1.5 µM Cas9 protein (IDT, Alt-R S.p. Cas9 nuclease V3, 1081061), 1.8 µM tracrRNA (Alt-R CRISPR-Cas9 tracrRNA, 224102825), 1.8 µM *Tcf7* AC crRNA (3068068) or CD5 crRNA (2997292), 1.8 µM electroporation enhancer (Alt-R Cas9 electroporation enhancer) and 300,000 cells per transfection. Transfection was performed using the NEON transfection instrument (ThermoFisher, settings: 1600V, 10ms pulse width, 3 pulses). After electroporation cells

were cultured for 3-4 days at 37°C with anti-mouse CD3/28 microbeads and IL-2 (Novartis, Proleukin S, 500 units/ml).

CRISPR/Cas9-mediated deletion of TCF1 in human T cells

Human peripheral blood was separated by Ficoll gradient centrifugation and pre-enriched with anti-human CD4 biotinylated antibody (Biolegend Clone OKT-4) and anti-biotin beads (Miltenyi Biotec). Cells were purified using magnetic columns (Miltenyi Biotec), followed by fluorescence-activated cell sorting (FACS) for $CD4^+CD25^-CD127^+$ T_{conv}. Cells were activated with anti-human Transact (Miltenyi 130-111-160; 1µl/well) and IL-2 (Novartis, Proleukin S, 500 units/ml) for 3 days in serum-free medium (TexMACS™, Miltenyi Biotec #130-097-196). Knock-down was performed with 1.5 µM Cas9 protein (IDT, Alt-R S.p. Cas9 nuclease V3, 1081061), 1.8 µM tracrRNA (Alt-R CRISPR-Cas9 tracrRNA, 224102825), 1.8 µM *Tcf7* AA crRNA (299900012) or *Tcf7* AC crRNA (299900014), or control crRNA (224509651), 1.8 µM electroporation enhancer (Alt-R Cas9 electroporation enhancer) and 300,000 cells per transfection. Transfection was performed using the NEON transfection instrument (ThermoFisher, settings: 1600V, 10ms pulse width, 3 pulses). After electroporation cells were cultured for 4 days at 37°C with anti-human Transact and IL-2 (Novartis, Proleukin S, 500 units/ml).

CRISPR/Cas9-mediated deletion of TCF1 in human T cells followed by stimulation and intracellular cytokine secretion analysis

$CD4^+CD25^-CD127^+$ T_{conv} cells were sorted and human TCF1 was deleted as described above. Then, T cells were treated with a cell stimulation cocktail plus transport inhibitor (eBiosciences # 00-4975-03) for 4 hours at 37°C. Afterwards, cells were stained for

intracellular cytokine expression with a PE-conjugated anti-human IL-2 antibody (MQ1-17H12) and the Foxp3 transcription factor buffer set (eBiosciences 00-5523-00).

Flow cytometry of T cells

Spleen, lymph node or thymus were cut into small pieces and mechanically mashed using filters and syringe plumbers. Red blood cells were lysed using commercially-available ACK lysis buffer. Murine cells were surface-stained for 20 minutes at 4°C with anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD25 (PC61), anti-CD90.1 (OX-7), anti-CTLA4 (UC10-4B9) and a fixable live/dead dye (eBiosciences 65-0865-18). Human cells were surface-stained for 20 minutes at 4°C with anti-CD4 (OKT-4) and a fixable live/dead dye (eBiosciences 65-0865-18). Cells were fixed and permeabilized using the Foxp3 transcription factor buffer set (eBiosciences 00-5523-00). Intracellular staining was performed for 60 minutes at RT with anti-mouse Foxp3 (JFK-16) or anti-human Foxp3 (206D) antibody at 1:100 dilution in Perm buffer. Anti-mouse/human Tcf1 antibody (C63D9) and staining was performed for 60 minutes at RT at 1:100 dilution in Perm buffer, followed by washing steps and secondary intracellular staining with 1:200 anti-rabbit AF647 antibody (Molecular Probes A21244). Samples were acquired on BD LSRII, BD CantoII or BD FortessaII flow cytometers.

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