Supporting Information

Discovery of Small Molecules Targeting the Synergy of Cardiac Transcription Factors GATA4 and NKX2-5

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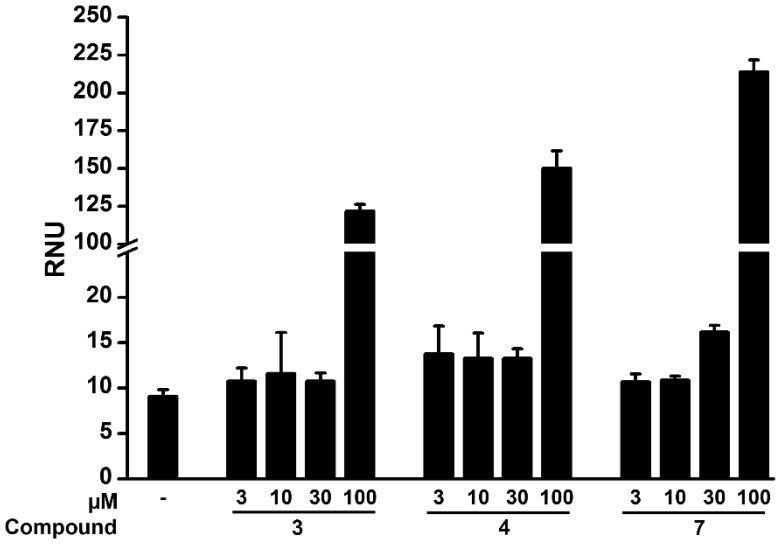
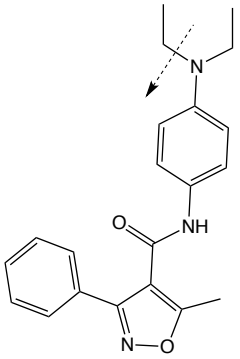


Figure S1. Aggregation of compounds. The blank and four concentrations of compounds **3**, **4**, and **7** were measured as triplicates at 400 V voltages using a Nepheloskan Ascent® (Labsystems). At concentrations up to 30 μM, the values were close to those in the control samples, indicating that there is no detectable aggregation, except for compound **7**, which demonstrated minor aggregation. RNU = relative nephelometric unit. The data are shown as the mean ± SD.

Table S1. Chemical stability of compound **3** was studied for 5 and 10 days in mouse embryonic stem cells (mESCs) *in vitro* at two different concentrations (3 and 5 μM) with two blank treatments (DMSO and embryoid body differentiation medium (EBDM)) added to the cell culture media. The intra- and extracellular concentrations of compound **3** and metabolite **31** were measured in 16 samples by HPLC/MS after the specific sample pretreatment and extraction procedures were performed. The results demonstrate that compound **3** modestly degraded over the 10 days of the cellular assay.



Nro.	**Sample**			**Compound 3 Area**	**Metabolite Area**	**322/350**
	m/z			**350.18**	**322.16**	**%**
	Retention time (min)			**1.74**	**1.68**	
1	5 Days	intracellular	3 μM	109.332	0.68	0.6
2	10 Days	intracellular	3 μM	437.191	8.53	2.0
3	5 Days	intracellular	5 μM	203.819	2.18	1.1
4	10 Days	intracellular	5 μM	486.787	2.13	0.4
5	5 Days	extracellular	3 μM	2654.775	28.61	1.1
6	10 Days	extracellular	3 μM	2800.877	405.40	14.5
7	5 Days	extracellular	5 μM	3348.705	22.89	0.7
8	10 Days	extracellular	5 μM	3793.717	106.56	2.8

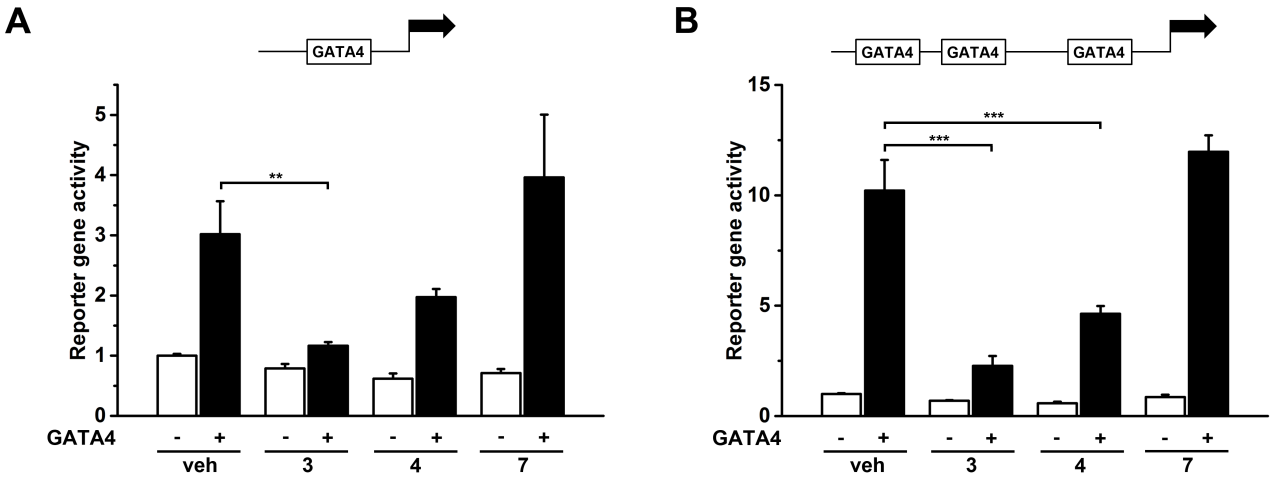


Figure S2. The effect of compounds **3**, **4**, and **7** on GATA4 transcriptional activity. Compounds were tested in COS-1 cells in a reporter assay by using BNP reporter constructs that are activated by GATA4. Compound **3** significantly inhibited GATA4 driven transactivation of both luciferase reporter constructs containing either BNP minimal promoter (A) or BNP promoter containing minimal promoter and tandem GATA-site on -90 bp (B). Compound **4** showed similar tendency, yet a statistically significant inhibition of gene transactivation was seen only with construct containing both minimal promoter and tandem GATA-sites (B). The data are shown as the mean ± SD, n = 3. ** $p<0.01$, *** $p<0.001$ vs. vehicle treatment.

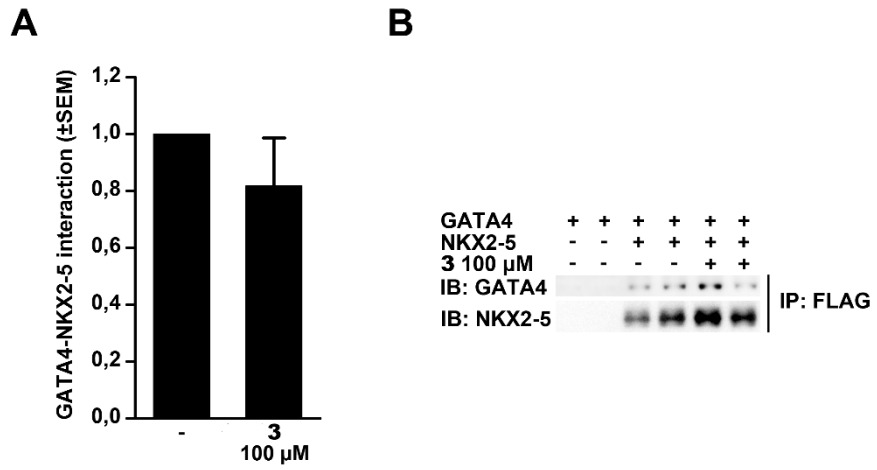


Figure S3. The effect of compound **3** on GATA4-NKX2-5 interaction in coimmunoprecipitation assay. GATA4 and NKX2-5-FLAG proteins were overexpressed in COS-1 cells and co-precipitated by anti-FLAG agarose. Compound **3** decreased about 20 % GATA4-NKX2-5 interaction at a concentration of 100 μM. The results are presented as mean of three independent experiments with two or three replicates ± SEM, n = 7.

Table S2. *In vitro* inhibition of protein kinases by compound **3** at a concentration of 30 μM as determined by the Cerep ExpresS Diversity Kinase Profile. 100% represents the full inhibition of the enzyme.

Kinase	% Inhibition	Kinase	% Inhibition
Abl	-4	JNK1	0
Akt1/PKBα	-9	KDR	64
AurA/Aur2	-5	Lck	10
CaMK2α	-5	MAPKAPK2	17
CDC2/CDK1	-3	MARK1	-6
CDK2	-1	MKK6	-3
CHK1	2	MNK2	-2

CHK2	-3	MST4	-1
CK1α	0	NEK2	8
c-Met	10	p38α	-6
EGFR	54	PAK2	2
EphA2	-2	PAK4	3
EphA3	-14	PDK1	-6
EphB4	17	Pim2	-2
ERK2	-13	PKA	2
FGFR1	2	PKCβ2	0
FGFR2	-5	PLK1	6
FGFR3	-10	RAF-1	6
GSK3β	3	ROCK1	-1
HGK	10	SGK1	-5
IKKα	-1	SIK	-1
IRAK4	-23	Src	-13
IRK	1	TAOK2	7
JAK3	0	TRKA	6

Abbreviations: Abl: Abelson murine leukemia viral oncogene homolog; Akt1/PKBα: Ak strain transforming kinase 1/Protein kinase B alpha; AurA/Aur2: Aurora kinase A; CaMK2α: Calcium/calmodulin-dependent protein kinase II alpha; CDC2/CDK1: Cell division cycle protein 2/Cyclin-dependent kinase 1; CDK2: Cyclin-dependent kinase 2; CHK1: Checkpoint kinase 1; CHK2: Checkpoint kinase 2; CK1α: Casein kinase I isoform alpha; c-Met: Hepatocyte growth factor receptor; EGFR: Epidermal growth factor receptor; EphA2: Ephrin type-A receptor 2; EphA3: Ephrin type-A receptor 3; EphB4: Ephrin type-B receptor 4; ERK2: Extracellular signal–regulated kinase 2; FGFR1: Fibroblast growth factor receptor 1; FGFR2: Fibroblast growth factor receptor 2; FGFR3: Fibroblast growth factor receptor 3; GSK3β: Glycogen synthase kinase 3 beta; HGK: Hepatocyte progenitor kinase-like kinase; IKKα: IκB kinase α; IRAK4: interleukin-1 receptor-associated kinase 4; IRK: insulin receptor kinase; JAK3: Janus kinase 3; JNK1: c-Jun N-terminal protein kinase 1; KDR: Kinase Insert Domain Receptor; Lck: Lymphocyte-specific protein tyrosine kinase; MAPKAPK2: Mitogen-activated protein kinase-activated protein kinase 2; MARK1: Microtubule affinity-regulating kinase 1; MKK6: Mitogen-activated protein kinase kinase 6; MNK2: Mitogen activated protein kinase-interacting protein kinase 2; MST4: Mammalian STE20-like protein kinase 4; NEK2: Never In Mitosis Gene A -related kinase 2; p38α: p38 mitogen-activated protein kinase alpha; PAK2: p21 activated kinase 2; PAK4: p21 activated kinase 4; PDK1: 3-Phosphoinositide-dependent protein kinase-1; Pim2: Proviral Integrations of Moloney virus 2; PKA: Protein kinase A; PKCβ2: Protein kinase C beta 2; PLK1: Polo-like kinase 1; RAF-1: Rapidly Accelerated Fibrosarcoma 1 kinase; ROCK1: Rho-associated, coiled-coil-containing

protein kinase 1; SGK1: Serum and glucocorticoid-regulated kinase 1; SIK: Salt-inducible kinase; Src: Schmidt-Ruppin A-2 viral oncogene homolog; TAOK2: Thousand and one amino acid protein kinase 2; TRKA: Tropomyosin receptor kinase A.

Table S3. *In vitro* inhibition of protein kinases by compound **4** at a concentration of 30 μM as determined by the Cerep ExpresS Diversity Kinase Profile. 100% represents the full inhibition of the enzyme.

Kinase	**% Inhibition**	**Kinase**	**% Inhibition**
Abl	14	JNK1	5
Akt1/PKBα	-8	KDR	10
AurA/Aur2	-19	Lck	8
CaMK2α	-3	MAPKAPK2	1
CDC2/CDK1	14	MARK1	-5
CDK2	29	MKK6	-2
CHK1	4	MNK2	-5
CHK2	3	MST4	-1
CK1α	-1	NEK2	-10
c-Met	12	p38α	-20
EGFR	11	PAK2	-92
EphA2	32	PAK4	0
EphA3	-5	PDK1	-3
EphB4	12	Pim2	0
ERK2	7	PKA	5
FGFR1	6	PKCβ2	1
FGFR2	7	PLK1	16
FGFR3	55	RAF-1	11
GSK3β	23	ROCK1	3

HGK	0	SGK1	1
IKKα	-1	SIK	3
IRAK4	21	Src	-26
IRK	-3	TAOK2	6
JAK3	4	TRKA	8

Abbreviations: See Table S2.

Table S4. The effect of compound **3** on different G-protein coupled receptors was assessed by Millipore GPCR Profiler. Percentage activation and percentage inhibition values were determined for each compound assayed at the concentrations of 12.5 μM and 10 μM, respectively. ND, not determined.

GPCR Target	**Agonism (%)**	**Antagonism (%)**	**GPCR Target**	**Agonism (%)**	**Antagonism (%)**
5-HT1A	-5.3	-13.8	GPR120	-0.1	ND
5-HT2A	-1.8	20.3	GPR14	0.0	-1.7
5-HT2B	-0.1	15.3	GPR39	-1.8	-1.3
5-HT2C	-2.2	8.8	GPR40	0.0	-0.7
5-HT6	-1.0	20.2	GPR41	0.0	3.3
A1	-0.3	0.5	GPR43	-0.6	9.1
A2A	1.4	4.5	GPR54	-0.7	7.7
A2B	-3.7	-5.5	GPR68	6.3	0.4
A3	-0.3	8.2	GPR91	0.3	1.5
ADRA1A	-0.4	2.4	GPR99	-0.7	26.0
ADRA1B	-1.0	0.7	H1	-1.0	5.9
ADRA1D	-2.6	11.2	H2	-1.0	-0.3

ADRA2A	0.0	9.0	H3	-1.7	-4.6
ADRA2B	-2.8	ND	IP1	0.0	1.2
ADRA2C	-3.4	9.6	LH	-0.2	14.8
ADRB1	-0.8	1.3	LPA1	-0.4	13.6
ADRB2	-0.5	9.3	LPA2	-0.2	11.0
ADRB3	-0.1	-11.3	LPA3	0.0	-16.7
APJ	-1.5	8.9	LPA5	0.4	7.7
AT1	-0.4	13.9	M1	-0.3	13.0
BB1	-0.2	1.4	M2	-0.5	16.1
BB2	-0.4	5.8	M3	-1.5	12.8
BB3	-3.6	-6.0	M4	0.0	15.9
BDKR2	-0.4	9.2	M5	-0.6	7.1
BLT1	-0.2	7.5	MC2	-0.7	5.0
C3aR	-0.5	4.3	MC4	-0.8	2.0
C5aR	-2.9	-1.2	MC5	-0.1	2.3
CaS	-0.4	-28.2	MCHR1	-0.4	1.6
CB1	-1.1	19.2	MCHR2	-0.8	3.0
CB2	-1.9	91.8	mGlu1	-0.2	1.2
CCK1	-0.2	-1.2	mGlu2	-0.5	16.2
CCK2	-0.3	5.5	Motilin	-0.5	18.3
CCR1	-0.5	3.9	MrgD	-0.1	6.2
CCR10	0.0	2.4	MRGX1	-5.7	17.1
CCR2B	0.4	1.6	MRGX2	-0.8	8.1
CCR3	-0.4	6.8	NK1	-1.2	3.9
CCR4	0.6	8.4	NK2	-0.4	5.8

CCR5	-0.1	6.3	NK3	-0.2	6.7
CCR6	-1.4	13.3	NMU1	-1.0	13.7
CCR7	-0.2	2.9	NMU2	-0.5	-2.5
CCR8	-2.1	3.9	NOP	-1.2	20.6
CCR9	0.4	-5.3	NPBW1	-0.4	1.9
CGRP1	-0.4	-4.9	NTR1	-1.0	12.9
ChemR23	-0.7	-2.9	OPRD1	-0.1	24.3
CRF1	-0.2	-0.8	OPRM1	-0.9	9.9
CRF2	-0.1	1.5	OT	0.4	5.9
CX3CR1	0.7	-12.4	OX1	-0.9	40.1
CXCR1	-1.0	8.3	OX2	3.3	9.9
CXCR2	-0.8	-3.4	P2Y1	-1.5	-27.0
CXCR3	0.2	4.8	P2Y11	-0.1	-3.4
CXCR4	-0.3	-4.0	P2Y2	-2.8	ND
CXCR5	-2.3	-7.6	P2Y4	-1.2	-2.6
CXCR6	-0.7	10.4	PAC1	-0.9	11.5
CysLT1	-0.6	2.6	PAF	-0.5	17.0
CysLT2	0.1	-3.3	PK1	-0.4	-19.3
D1	0.8	5.9	PK2	0.9	-6.9
D2	-0.9	-12.4	PRP	-0.3	9.0
D4	-0.2	15.2	PTH1	-0.9	-0.8
D5	2.1	26.5	PTH2	-1.6	59.5
DP	-3.7	13.4	S1P1	-0.1	0.6
EP1	0.7	3.9	S1P2	-0.7	12.6
EP2	-0.3	3.1	S1P3	-0.2	14.1

EP3	-0.3	3.6	S1P4	-2.1	9.4
EP4	-1.4	10.0	S1P5	0.5	-1.4
ETA	-0.8	4.4	Secretin	-0.7	16.0
ETB	0.1	1.5	SST2	-0.8	6.4
FP	-0.3	12.9	SST3	-0.7	17.1
FPR1	-0.3	-2.5	SST4	-0.8	-0.3
FPR2	-0.3	13.0	SST5	-3.4	-55.3
FSH	-0.8	5.2	Thrombin-activated PARs	-0.4	8.1
GABAB1b	-1.1	-41.8	TP	-0.7	-19.9
GAL1	0.6	10.6	TRH	-0.8	-0.8
GAL2	-0.3	9.8	Trypsin-activated PARs	0.0	9.4
GCGR	-0.9	-7.5	TSH	-0.3	21.1
Ghrelin	-1.0	-65.2	V1A	-0.4	9.2
GIP	-0.4	7.6	V1B	-0.8	14.1
GLP-1	-0.5	0.1	V2	-0.8	27.4
GLP-2	-1.0	-0.2	VPAC1	-0.7	-1.4
GnRH	0.0	-3.1	VPAC2	2.6	15.7
GPBA	-2.4	ND	XCR1	-0.2	-7.8
GPR103	-0.2	4.8	Y2	-2.7	3.8
GPR109	-0.5	58.5	Y4	-0.3	9.1
GPR119	4.1	ND			

Abbreviations: 5HT: 5-hydroxytryptamine receptor; A: Adenosine receptor; ADRA: Alpha-adrenergic receptor; ADRB: Beta-adrenergic receptor; APJ: Apelin receptor; AT1: Angiotensin II receptor, type 1; BB: bombesin receptor; BDKR: Bradykinin receptor; BLT1: Leukotriene B4 receptor 1; C3aR/C5aR: Complement Anaphylatoxin C3/C5a Receptor; CaS: Calcium-sensing receptor; CB: Cannabinoid receptor; CCK: Cholecystokinin receptor; CCR: Chemokine receptor; CGRP1: Calcitonin gene-related peptide 1 receptor; Chem23: Chemerin receptor 23; CRF: Corticotropin-releasing hormone receptor; CX3CR1: C-X3-C motif chemokine receptor 1; CXCR: C-X-C chemokine receptor; CysLT: Cysteinyl leukotriene receptor; D: Dopamine receptor; DP: D prostanoid receptor; EP: Prostaglandin E2 receptor; ET: Endothelin receptor; FP: Prostaglandin F receptor; FPR: Formyl peptide receptor; FSH: Follicle-stimulating hormone receptor; GABAB1b: Gamma-aminobutyric acid B type 1b receptor; GAL: Galanin receptor; GCGR: Glucagon receptor; GIP: Gastric inhibitory polypeptide receptor; GLP: Glucagon-like peptide receptor; GnRH: Gonadotropin-releasing hormone receptor; GPBA: G protein-coupled bile acid receptor; GPR: G protein-coupled orphan receptor; H: Histamine receptor; IP: Prostacyclin receptor; LH: Luteinizing hormone receptor; LPA: Lysophosphatidic acid receptor; M: Muscarinic acetylcholine receptor; MC: Melanocortin receptor; MCHR: Melanin-concentrating hormone receptor; mGlu: Metabotropic glutamate receptor; MrgD: MAS-related G protein-coupled receptor, member D; MRGX: Mas-related G protein-coupled receptor member X; NK: Neurokinin receptor; NMU: Neuromedin U receptor; NOP: Nociceptin receptor; NPBW: Neuropeptides B/W receptor; NTR: Neurotrophin receptor; OPRD: δ-opioid receptor; OPRM: μ-opioid receptor; OT: Oxytocin receptor; OX: Orexin receptor; P2Y: Purinergic G protein-coupled receptor; PAC1: Pituitary adenylate cyclase-activating polypeptide type I receptor; PAF: Platelet-activating factor receptor; PK: Prokineticin receptor; PRP: Prolactin-releasing peptide receptor; PTH: Parathyroid hormone receptor; S1P: Sphingosine-1-phosphate receptor; SST: Somatostatin receptor; PAR: Protease-activated receptor; TSH: Thyrotropin receptor; V1: Vasopressin receptor; VPAC: Vasoactive intestinal peptide receptor; XCR: X-C motif chemokine receptor; Y: Neuropeptide Y receptor. ND: not determined.

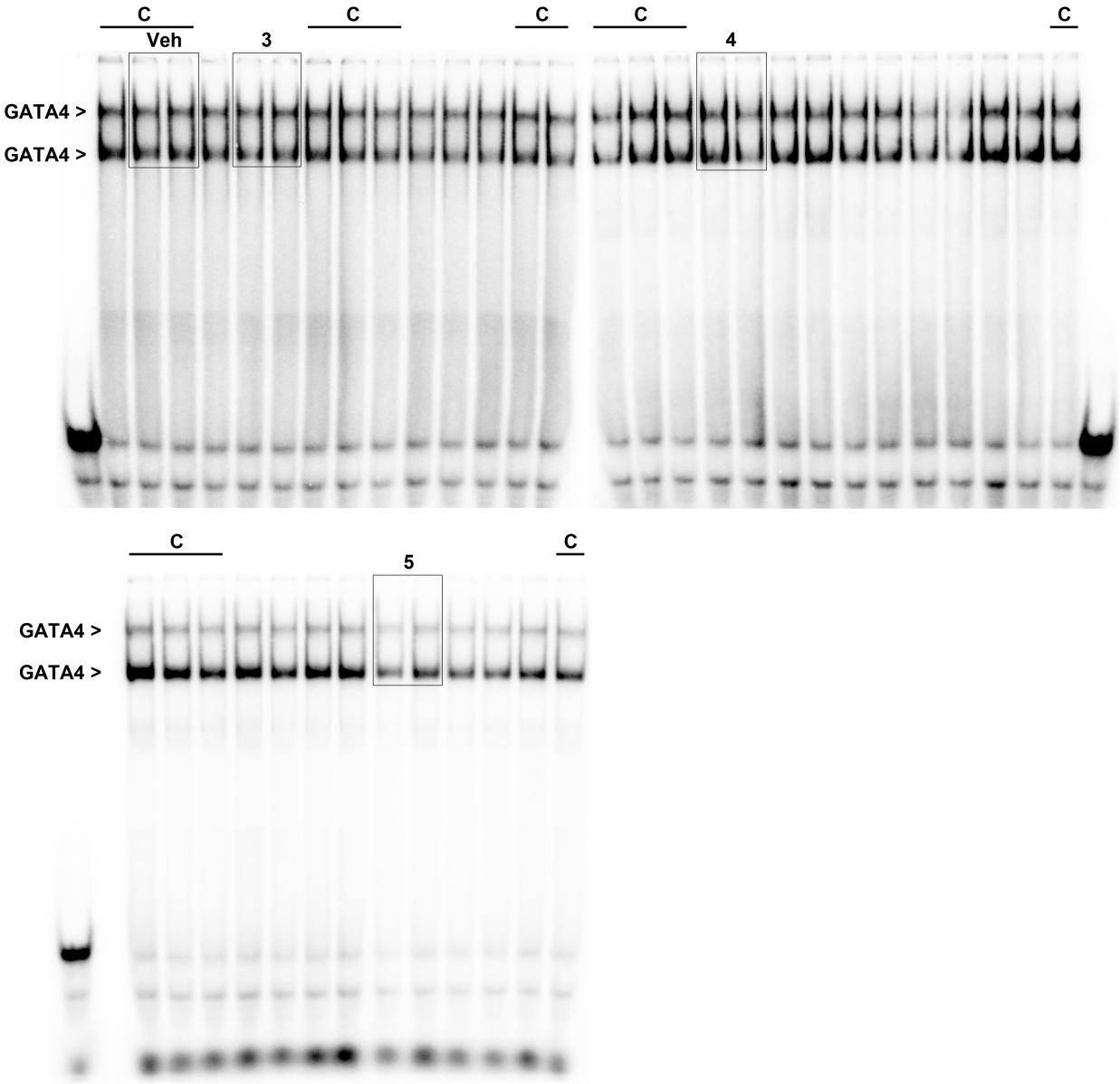


Figure S4. Original EMSA gels for the Figure 3A. The bands shown in Figure 3A are circled by a rectangle and other extra lanes were removed. The bands were normalized towards the vehicle treated control samples (denoted as C) on the same gel.

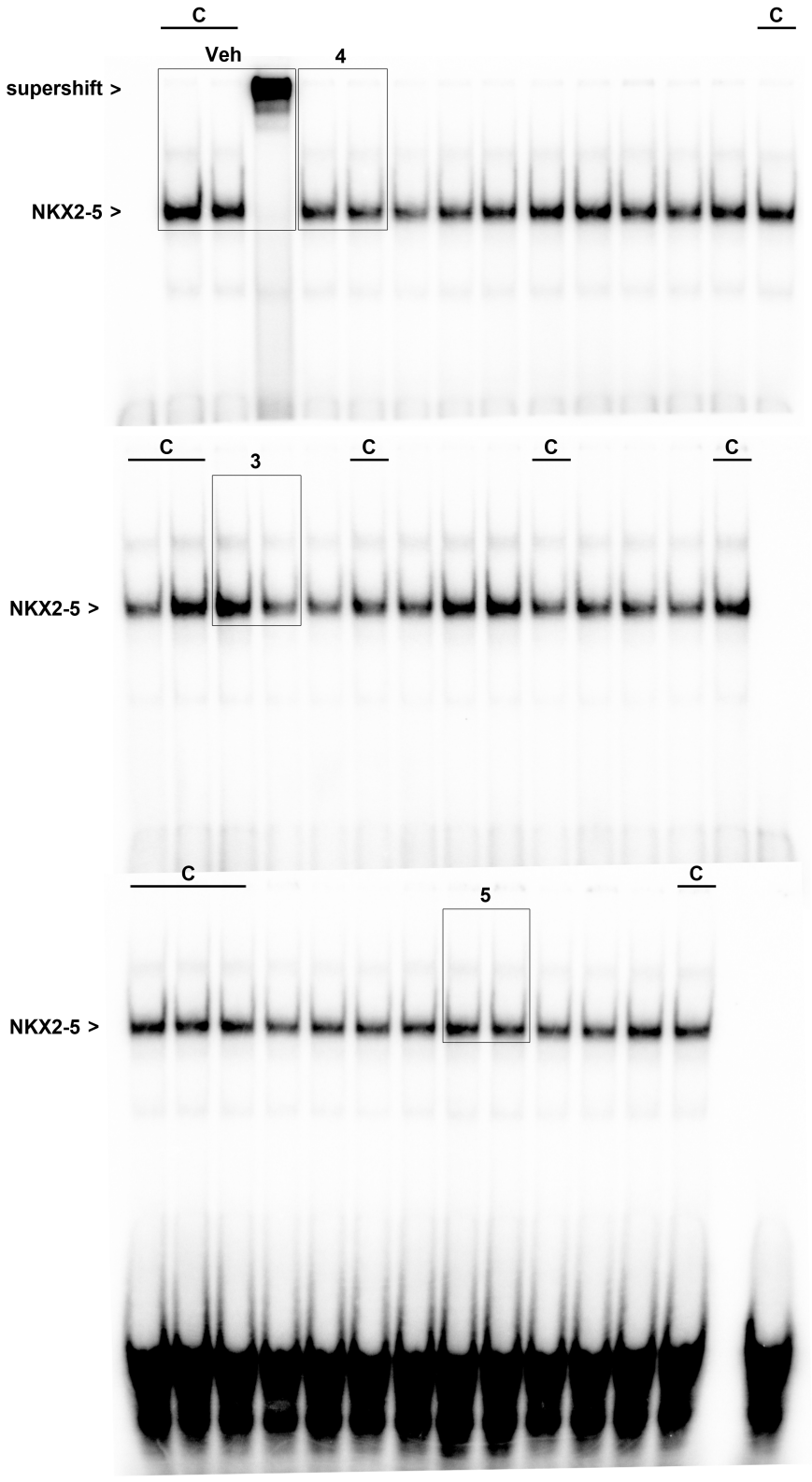


Figure S5. Original EMSA gels for the Figure 3B. The bands shown in Figure 3B are circled by a rectangle and other extra lanes were removed. The bands were normalized towards the vehicle treated control samples (denoted as C) on the same gel.

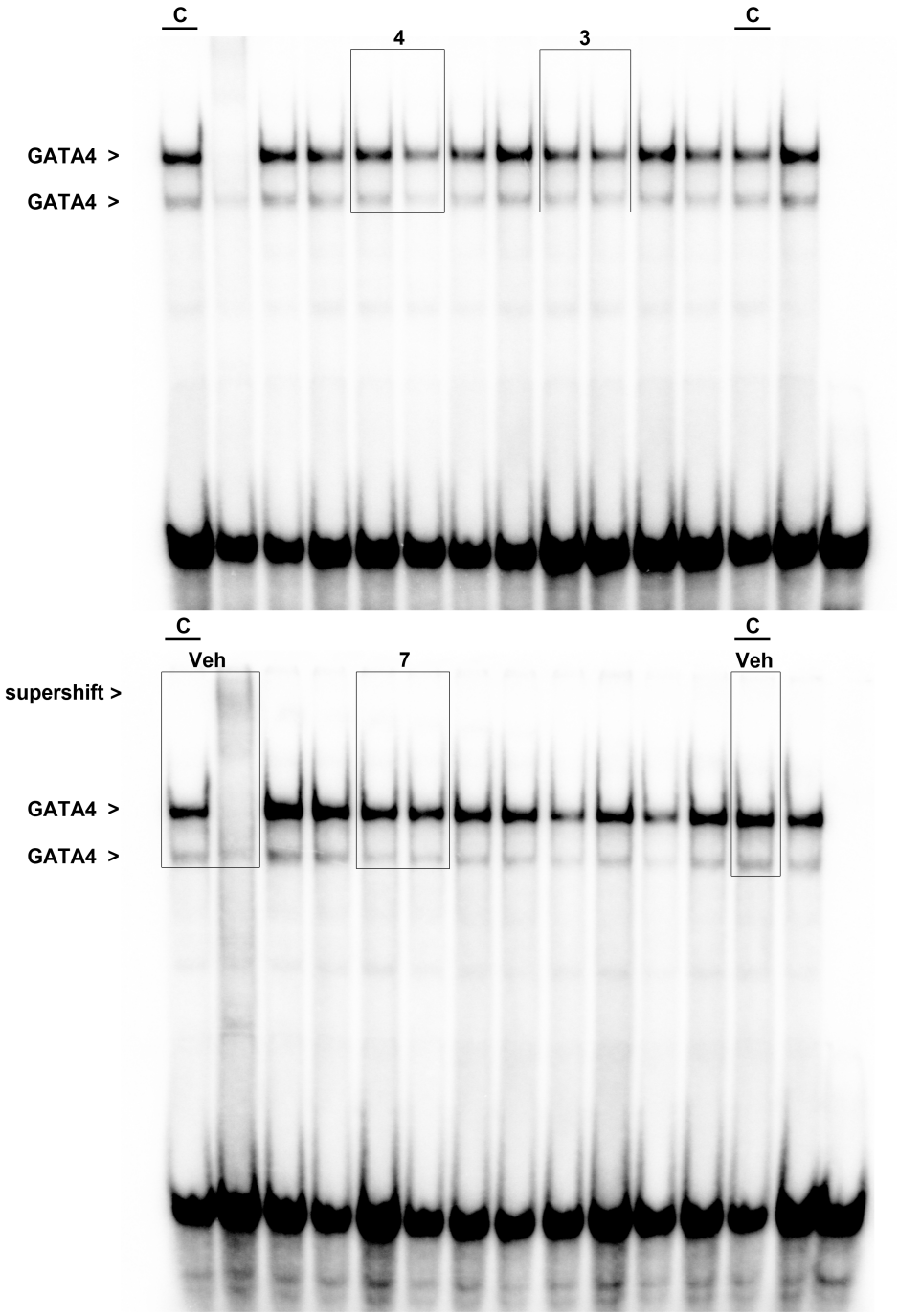


Figure S6. Original EMSA gels for the Figure 3C. The bands shown in Figure 3C are circled by a rectangle and other extra lanes were removed. The bands were normalized towards the vehicle treated control samples (denoted as C) on the same gel.

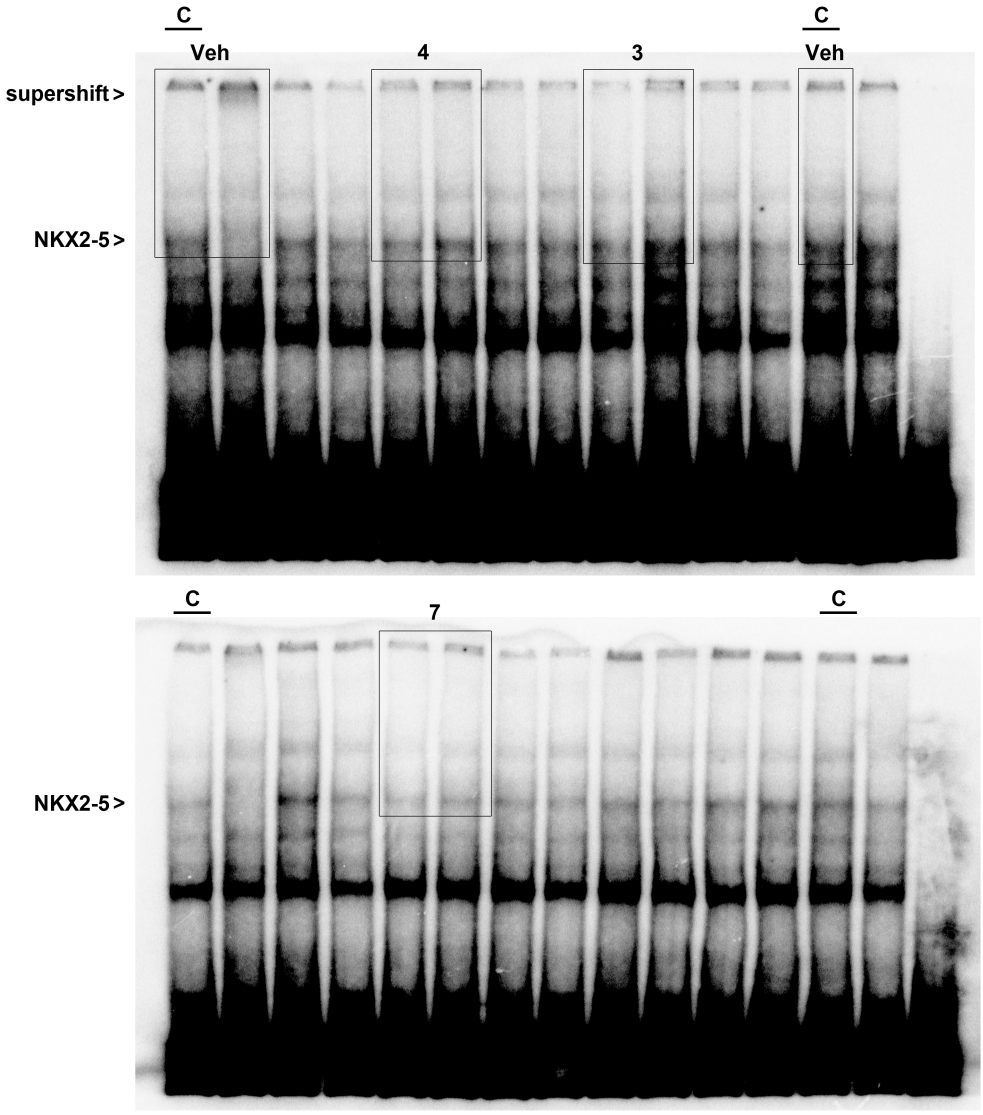


Figure S7. Original EMSA gels for the Figure 3D. The bands shown in Figure 3D are circled by a rectangle and other extra lanes were removed. The bands were normalized towards the vehicle treated control samples (denoted as C) on the same gel.