SUPPLEMENTARY INFORMATION

Monoclonal FoxO1 reporter cell lines

Various FoxO1 promoter sequences (Supplementary Figure 6A) ranging from 0.5 kb to 4.4 kb (Supplementary Figure 6B) were inserted to pGL4.16 [Luc2CP/Hygro] plasmid (Promega) which encodes the luciferase reporter gene luc2CP. This vector also contains a mammalian selectable marker for hygromycin resistance.

SW1353 chondrosarcoma cells were transfected with the plasmids containing FoxO1 promoter sequences using Lipofectamine 3000 (Invitrogen) and incubated with 400 μg/ml hygromycin to select only transfected cells. Some of the constructs contained the binding site of LXRα activator and we thus tested T0901317(TO9) (Sigma), a potent and selective agonist for both LXRα and LXRβ, with a half maximal effective concentration (EC_{50}) of about 50 nM[1, 2]. 5 -aminoimidazole -4 -carboxamide -1-β-d-ribofuranoside (AICAR)[2], and LY 294002, a PI3 kinase inhibitor (Cell Signaling) were also tested as they have been reported to induce FoxO1 mRNA expression. Cells were treated with these compounds for 24 hours, and luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega). Among the FoxO1 promoter constructs, the 0.8 kb or 1.6 kb constructs showed the highest luciferase activities of all the constructs. We thus prepared monoclonal SW1353 cell lines with the 0.8 kb or 1.6 kb FoxO1 promoter luciferase constructs by serial dilution methods, and then we compared the luciferase activity between the top 2 clones **(**Supplementary Figure 6B**)**. Clone FoxO1 0.8-3 showed the largest increase in luciferase activity and was selected for use in the high throughput drug screening (HTS) (Supplementary Figure 6C).

High throughput drug screening with ReFRAME library

To find compounds that upregulate FoxO1 expression for OA treatment, we used the Repurposing, Focused Rescue, and Accelerated Medchem (ReFRAME) Library for HTS. The ReFRAME library is composed of 11,948 small molecules that have reached clinical development or undergone significant preclinical profiling[3]. The drugs were pre-spotted into 1536-well assay plates with the Echo Acoustic Liquid Handler (Beckman) to achieve a final concentration of 5 μM in an 8 μL/well final assay volume. The monoclonal SW1353 cell line containing the FoxO1 0.8 kb promoter-luciferase construct was dispensed into the assay plates with 100 cells per well. Cells were allowed to grow for 24 h at 37°C with

5% CO_2 in a humidified tissue culture incubator. Luciferase activities were measured using Steady Glo (Promega) according to the manufacturer's instruction. The hit compounds were replated for confirmation assays first in triplicate and then evaluated in a 10-point dose-response concentration curve. EC_{50} were calculated from the dose-response curve, and the hit compounds were ranked from the lowest to highest EC_{50}.

In vitro experiments with human chondrocytes, synoviocytes and meniscus cells

Human primary OA chondrocytes, synoviocytes and meniscus cells were isolated from patients undergoing total knee arthroplasty and cultured as previously described[4],[5]. First passage cells were used for experiments. Normal human chondrocytes were isolated from normal human knee joints which were obtained from tissue banks and maintained in the same way. In all in vitro experiments, SW1353 human chondrosarcoma cells were plated at 5×10^3 cells /mL and human primary cells were plated at 1×10^4 cells/mL. In experiments where drugs were diluted with DMSO, the DMSO concentration in the culture medium was always adjusted to 0.1%.

Validation of selected hit compounds using SW1353, OA and normal human chondrocytes

To validate and compare the effect of hit compounds from the ReFRAME screen, compounds with suitable EC_{50} and biological properties that suggest potential benefit in OA were selected and tested in SW1353 cells, OA and normal chondrocytes. Cells were seeded in 96 well plates, cultured for 24 h, incubated with the selected compounds at 0.1×, 1× and 10× EC_{50} for SW1353 cells and at 1×, 5× and 10× EC_{50} for chondrocytes for 24 h, and then mRNA was extracted for RT-qPCR.

Dose effects of Panobinostat on cell viability and gene expression human chondrocytes, synoviocytes and meniscus cells with and without IL-1β stimulation

For cell viability experiments, OA chondrocytes precultured for 24 h and then incubated with various doses of Panobinostat for 24 h and cell viability was tested with CellTiter-Glo (Promega) according to the manufacturer's instruction.

To examine dose-dependent effects of Panobinostat on gene expression, OA chondrocytes, synovial cells, and meniscus cells were pre-cultured for 24 h and then incubated with various doses of Panobinostat for another 24 h with and without IL-1β (1 ng/ml) for additional 6 h.

Chondrocyte Treatment with FoxO1 inhibitorAS1842856.

To test which Panobinostat effects were dependent on FoxO1, chondrocytes were treated with Panobinostat (80 nM) in the presence or absence of the specific FoxO1 inhibitor AS1842856[6] (Sigma-Aldrich 344355; 0.1 μM) for 24 hours and RNA was isolated for qRT-PCR analysis.

RNA isolation and RT-qPCR

In cultured cells, mRNA was collected using Direct-Zol RNA miniprep kit (Zymo Research). Gene expression was measured by RT-qPCR using pre-designed TaqMan gene expression assays. GAPDH was measured as a reference gene.

Mice

All animal experiments were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee at Scripps Research. Mice from the C57BL/6J strain were used in all experiments.

Short-term effect of Panobinostat in vivo on normal mouse knee cartilage and IL-1β induced inflammation

To determine the short-term effects of Panobinostat on mouse knee cartilage without experimental OA, C57BL/6J mice (all male mice, age 4 months) received 3 intraperitoneal injections every other day of 100 μg/kg or 2.5 mg/kg of Panobinostat or DMSO vehicle (0.1%). Knee cartilage was collected 1 to 3 h after the final injection for RNA isolation.

We next investigated the in vivo effect of Panobinostat on IL-1β induced inflammation. Two hours after intraperitoneal injection of 2.5 mg/kg of Panobinostat or DMSO vehicle, 5 ng of IL-1β was injected into the right knee and the left knee was untreated as control knee. Total RNA of knee cartilage and synovium was collected 6 h after the IL-1β injection. The RT-qPCR values of right knee were normalized with left knee in each mouse, and we compared the average values between the vehicle-treated control group and the Panobinostat-treated group.

Panobinostat treatment of mice with surgically induced OA

For the surgical OA model, 4-month-old male C57BL/6J mice (n=14 per group) were anesthetized and transection of the medial meniscotibial ligament (DMM) was performed in the right knee as described[7,

[8]. To serve as control, sham surgery was performed on the left knee and consisted of a small incision on the medial side and opening of the joint capsule and closing with surgical suture. Animals were euthanized 8 weeks or 12 weeks after surgery, and knee joints were embedded in a standardized fixed angle of the femur relative to the tibia so that the sections that were scored represent the center of the weight-bearing areas of the tibial plateau and femoral condyle[9]. Five-μm thick sagittal sections of the medial compartment of the knee were stained with Safranin-O and fast green[9], and OA related changes were scored as previously described for articular cartilage (summed OARSI score for femur and tibia)[10], synovium[11], and subchondral bone[12]. Samples were graded by two different individuals blinded to the treatment. In the first experiment, we assigned three treatment groups, DMSO vehicle-treated group, 100 mg/kg and 2.5 mg/kg Panobinostat-treated groups. Panobinostat or vehicle were administrated intraperitoneally 3 times per week for 7 weeks, starting 1 week after the surgery.
In the second experiment, we assigned two treatment groups, DMSO vehicle-treated group and 1 mg/kg Panobinostat-treated groups. Panobinostat or vehicle were administrated intraperitoneally 3 times per week for 11 weeks, starting 1 week after the surgery.

Measurements of knee swelling in mice

The lateral diameter of the knee that underwent DMM surgery was measured by caliper before the surgery and 1-9 days after the surgery, and the difference between vehicle-treated and Panobinostat-treated groups was calculated.

Pain measurements in mice

The von Frey assay for evaluating mechanical allodynia was conducted at 4 weeks and 8 weeks after DMM surgery[13]. Mice were placed on a mesh metal platform and the plantar surface of the hind paw was touched with #4 (1 g) and #5 (2 g) von Frey filaments 5 times for each hind paw since we could not observe paw withdrawal by less than #4 filaments. The number of paw withdrawals out of 5 repetitions were counted.

Statistical analysis

Data are reported as the mean ± SD. Multiple group analysis was assessed by one-way analysis of variance (ANOVA) followed by a post-hoc Tukey test. Comparisons between two groups were assessed by an unpaired, two-tailed T-test after testing for equal variance using an F-test. All statistical analyses

were performed using Prism 6 software (GraphPad Software). P-values less than 0.05 were considered significant.

SUPPLEMENTAL FIGURE LEGENDS

Supplementary Figure 1. Dose response analysis of selected HDACi in counter-screen.

Supplementary Figure 2. Cell viability of chondrocytes after treatment with Panobinostat.

Supplementary Figure 3. Effects of FOXO1 inhibition on Panobinostat treatment.
Human normal healthy chondrocytes (passage 1 from 7 donors) were incubated with Panobinostat (80 nM) in the presence or absence of the specific FOXO1 inhibitor AS1842856 (0.1 μM) for 24 hours and RNA was isolated for qRT-PCR analysis.
****= $p<0.0001$; ***= $p<0.001$; **= $p<0.01$; *= $p<0.05$.

Supplementary Figure 4. Knee swelling of mice that were subjected to DMM.

Supplementary Figure 5. Von Frey testing for evaluating mechanical allodynia was performed with 2 g filaments at the indicated time points after DMM in the 8-week and 12-week Panobinostat treatment experiments.

Supplementary Figure 6. FoxO1 reporter constructs. (A) FoxO1 promoter. (B) Various FoxO1 promoter sequences ranging from 0.5 kb to 4.4 kb were inserted to pGL4.16 [Luc2CP/Hygro] plasmid (Promega) which encodes the luciferase reporter gene luc2CP.

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Suppl Table 1. Non-hit HDACI

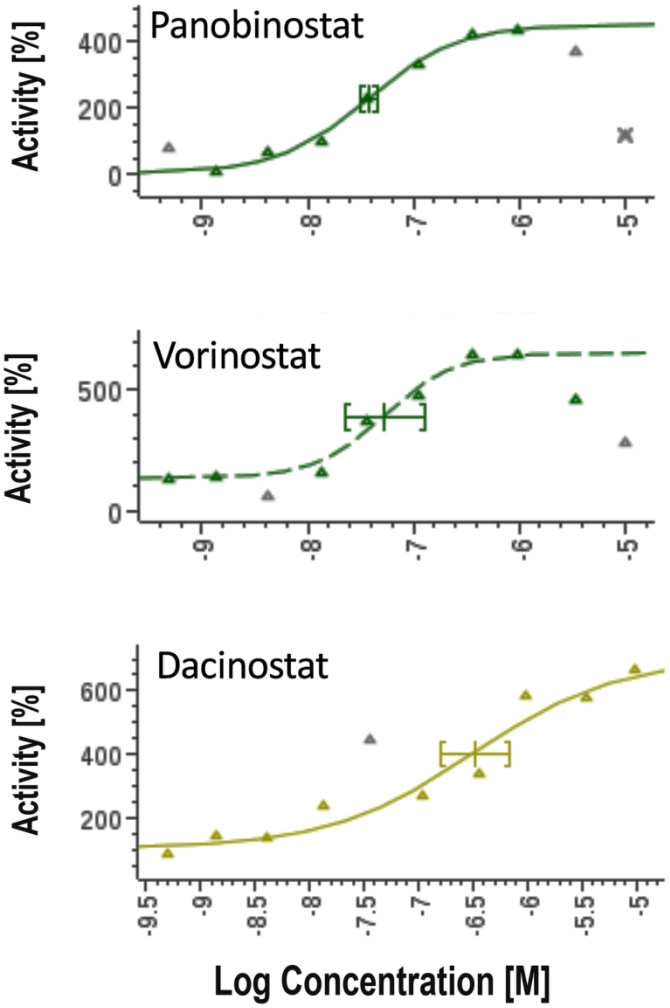
ISOVALERAMIDE
Mocetinostat
Ricolinostat
Resminostat (RAS2410)
Entinostat (MS-275)
Romidepsin
Sodium Phenylbutyrate (AMX0035)
VALPROIC ACID
Pivanex (AN-9)
R306465
CRA-026440
Nanatinostat (CHR-3996)
Chidamide
GLYCEROL PHENYLBUTYRATE
4SC-202
RG2833
Remetinostat
Fimepinostat (CUDC-907)
Dihydrocoumarin
MGCD-290
EVP-0334
ARGININE BUTYRATE
Tinostamustine (EDO-S101)
Citarinostat

Suppl Table 2. Characteristics of hit and non-hit HDACI.

Hit	**Hydroxamic acids**	**Panobinostat, Vorinostat, CUDC-101, Quisinostat, Pracinostat, Abexinostat, Givinostat, Belinostat, CHR-3996, Resminostat, Scriptaid**
	Benzamides	Tacedinaline
Non-hit	Hydroxamic acids	Ricolinostat, Ivaltinostat, Remetinostat
	Short-chain fatty acids	Sodium phenylbutyrate, Valproic acid, Pivanex
	Benzamides	Mocetinostat, Entinostat, 4SC202
	Cyclic tetrapeptides	Romidepsin

Suppl Table 3. Classes and IC_{50} of hit and non-hit HDACI.

	Compound	HDAC isoform, IC_{50} (nM)								
		1	2	3	8	4	5	7	9	6
Hit	Panobinostat	3	2	2	22	1	1	2	1	1
	Vorinostat	60	42	36	173	20	36	129	49	29
	Quisinostat	0.1	0.3	5	4	0.6	4	119	32	77
	Pracinostat	28	27	19	48	16	21	104	24	247
	Abexinostat	21	63	148	370	60	48	350	168	12
	Givinostat	133	293	136	837	>1000	532	524	512	312
Non-hit	Ricolinostat	58	48	51	100	>1000	>1000	>1000	>1000	5
	Citarinostat	35	45	46	137	>1000	>1000	>1000	>1000	3
	Mocetinostat	200	300	1700	>10000	>10000	>10000	>10000		>10000
	Entinostat	200	1200	2300	>10000	>10000		>10000	500	>10000
	Romidepsin	1	1	1	>1000	647	>1000	>1000	>1000	226



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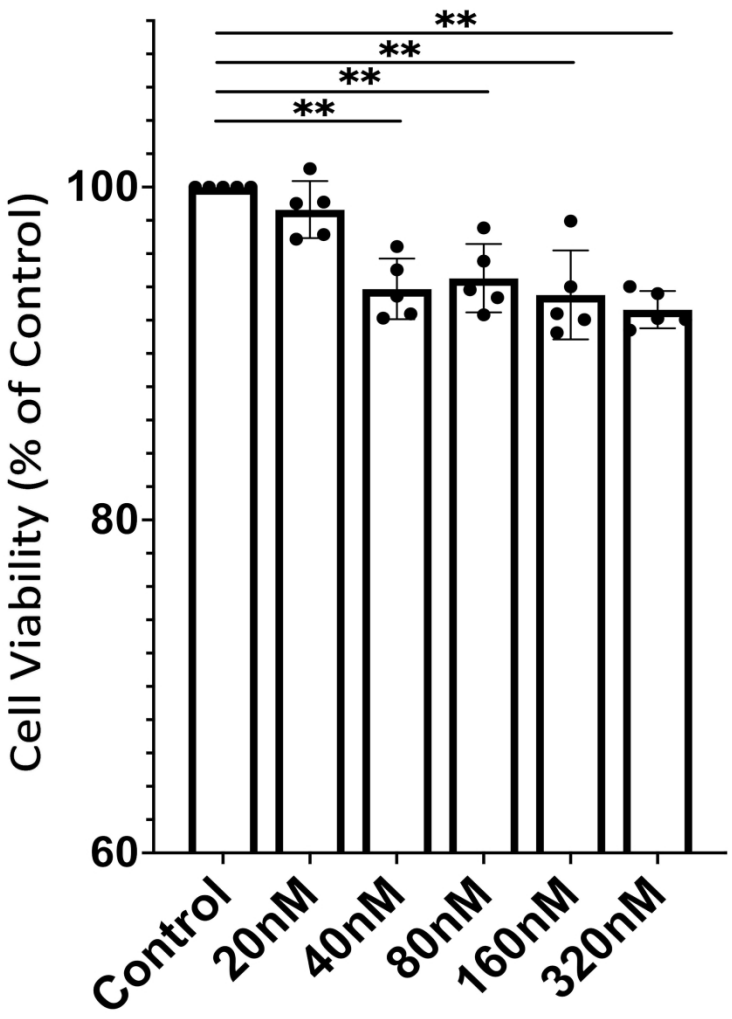

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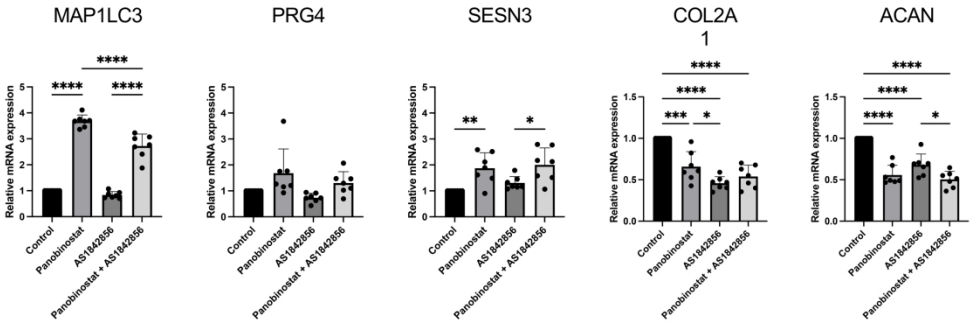

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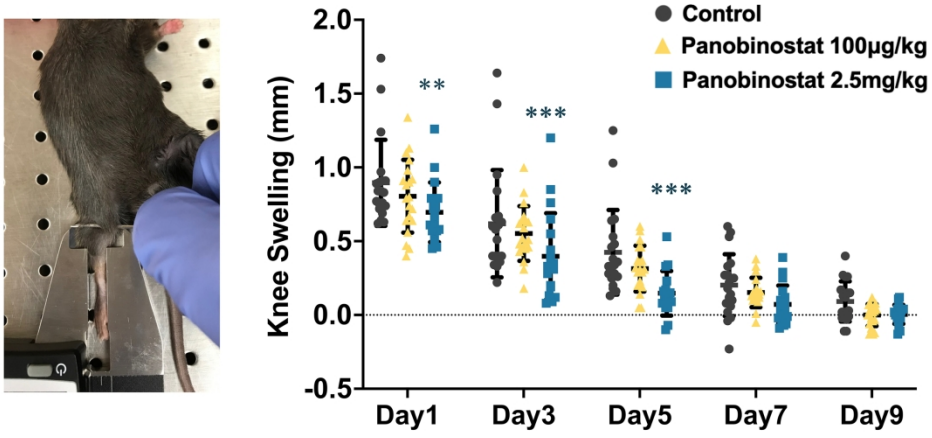

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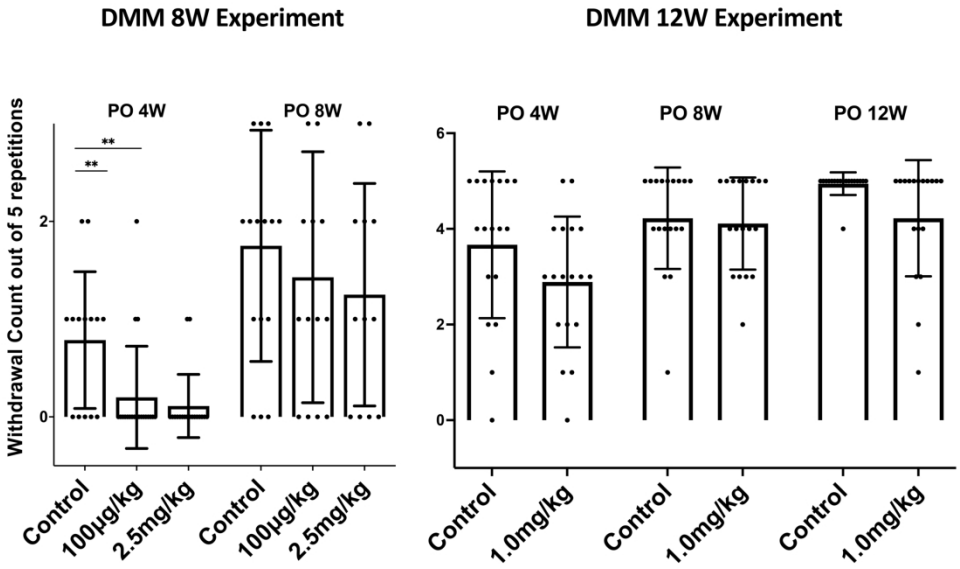

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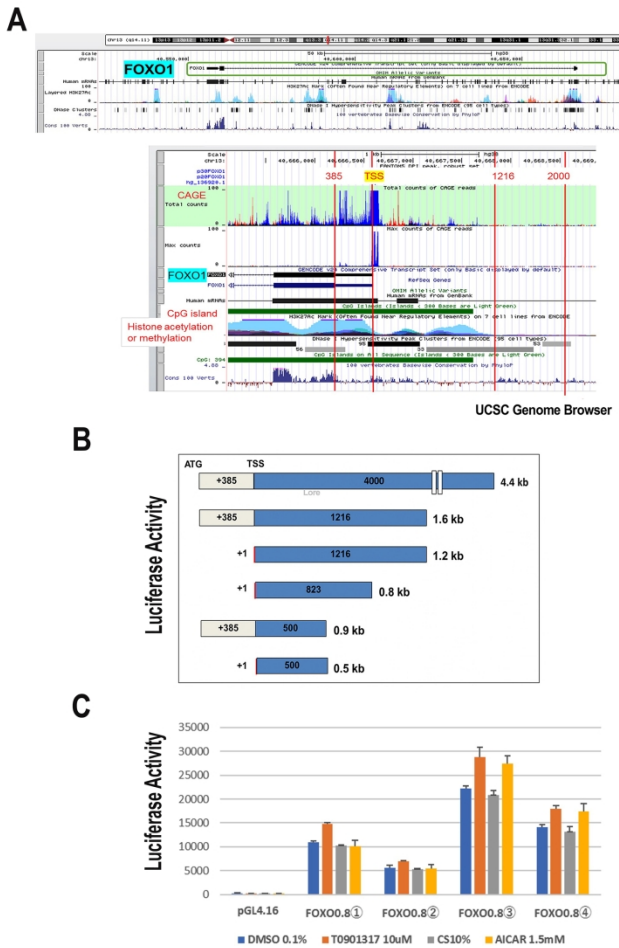

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