

Precision RNAi for Fibrodysplasia Ossificans Progressiva: a combinatorial, unimolecular, allele selective approach.

Corresponding Author: Professor JaeHyuck Shim

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The report by Yang et al describes the development of siRNAs to treat HO formation in FOP. They develop optimized siRNAs that selectively reduced ACVR1 R206H transcripts and couple them to a fatty acid to improve tissue distribution. In addition they develop an siRNA directed at IL1B and confirm that IL1B expression contributes to HO formation in FOP. Lastly they construct a lipid linked molecule consisting of siRNAs targeting both the ACVR1 mutation and IL1B. They show that both as a therapeutic and a prophylactic the dual targeting siRNA exhibits efficacy, albeit modest.

This is a beautifully crafted report. It is clearly written with extensive experimental support Yang et al make a very compelling case for evaluation of their siRNA constructs in human clinical trials. I liked the proposal that this agent be considered in the setting of surgery, since it could be introduced into the surgical field, reducing concerns regarding biodistribution and systemic toxicology.

Reviewer #2

(Remarks to the Author)

This manuscript reports the establishment of a safe and efficient treatment regimen for FOP, a rare disease characterized by the formation of bone in muscle (heterotopic ossification, HO), using a modified siRNA. The authors designed siRNA specifically targeting a mutant form of Acvr1, a causative gene for FOP, and conjugated it to docosanoic acid (DCA) to deliver it to muscles. They found that IL1B is one of the most robustly upregulated genes upon HO induction in mice, and targeted both mutant Acvr1 and IL1B simultaneously to demonstrate robust suppression of HO in the animal model. Their therapeutics are effective in both local (transdermal) and systemic injection.

This is a well-written manuscript supported by numerous data points with high clinical relevance. FOP is a devastating, irreversible disease, and despite recent huge efforts to develop therapeutics, it is still an urgent unmet clinical need to identify safer and more efficient treatment options. The authors' group has a track record of siRNA design. It is quite impressive that siRNA can stay in tissues for multiple weeks after a single injection. They used a transdermal microneedle system (microjet) to minimize muscle trauma, which can trigger HO for patients with FOP, though the feasibility and limitations of this transdermal approach in larger animals merit discussion. Validation of siRNA constructs and biological alterations in mice and human iPS cells from patients is robust and well supports their conclusions. This reviewer, however, has a couple of comments for clarity.

1. In Fig 3c and other places, dual silencing of Acvr1 and Ilb1 shows a reduction of HO volumes. In Fig 5a, the authors claimed dual silencing can restore muscle regeneration. Compared with Acvr1-only or Ilb1-only treatment, does the dual case show better regeneration?
2. In Fig 3d, why did the authors measure Inhba (encoding Activin A), and how can we explain its upregulation in the FOP model compared with the control condition?
3. In Fig 4, unconjugated siRNA accumulated in the kidney after 1 month of transdermal injection, but not in the liver. Compared with DCA conjugation, is this a general aspect of siRNA? The authors could mention the prevention of kidney accumulation as a benefit of DCA conjugation.

4. As the authors briefly mentioned, transdermal routes may have a limitation when applied to large animals. They could discuss the feasibility and limitations of the transdermal approach using a microjet, based on their previous data, and clarify how this delivery strategy may translate to other models.

5. How do they differentially quantify wild-type and mutant *Acvr1* using QRT-PCR?

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This manuscript describes an allele-selective siRNA (siACVR1) that discriminates the FOP-causing ACVR1R206H transcript from the wild-type allele, a second siRNA targeting *Il1b*/IL1B, and a unimolecular divalent construct (siACVR1_II1b) combining both activities in a single DCA-lipid-conjugated molecule. The authors provide extensive in vitro screening, allele-selectivity validation in patient-derived iPSCs and ex vivo human muscle, in vivo efficacy in a trauma-induced heterotopic ossification (HO) mouse model via local and systemic delivery, mechanistic RNA-seq in FACS-sorted FAPs, and initial safety/toxicology data.

This is an ambitious, largely well-executed body of work and, to my knowledge, the first allele-selective RNAi strategy for FOP validated across cell, tissue, and whole-animal levels, with a thoughtful unimolecular solution for co-delivering two siRNA activities. The ex vivo human muscle biopsy data (Extended Fig. 20) are a particular strength at this stage of preclinical development.

The core claims are generally well supported. In general, this manuscript presented nice quality while there are some critiques that need to be addressed before publication:

1) It seems the effectiveness of one dose can last 4-8 weeks, which are reasonably good for rodents. However, for clinical translation, repeating doses are likely necessary. Therefore, it is important to identify toxicity and immunogenicity of repeat doses. Ideally, add a short repeat-dose pilot with corresponding toxicology to strengthen the safety package in Figure 6/Extended Fig. 18–19.

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6) The pathway-level data (Extended Fig. 5: TGF β /3TP-Luc, WNT3a/Topflash, FGF2/pERK) are reassuring but indirect. ACVR1 belongs to a family of structurally related type I receptors (ACVR1B/ALK4, ACVR1C/ALK7, BMPR1A/ALK3, BMPR1B/ALK6) that could share partial complementarity with the guide strand outside the R206H hotspot. A direct mRNA-level knockdown panel against these paralogs—analogue to the TGFBR/WNT/FGFR pathway output already tested—would support the specificity claim more rigorously than reporter assays, which may miss partial (20–30%) reductions in receptor expression.

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8) A detailed discussion on the design and selection of the allele-selective siRNA would be helpful for the readers to understand a general strategy in siRNA design. This is an important piece of value of this work.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career

Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The authors substantially and appropriately responded to comments/suggestions from this reviewer and others.

Reviewer #3

(Remarks to the Author)

The revised manuscript has adequately addressed my critiques/comments.

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➤ No reviewer comments were left for this revision.

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- We thank the reviewer for the positive feedback and for recognizing the significance of our manuscript. We appreciate the constructive suggestions, which we believe have helped improve and strengthen the revised version.

1. In Fig 3c and other places, dual silencing of Acvr1 and Ilb1 shows a reduction of HO volumes. In Fig 5a, the authors claimed dual silencing can restore muscle regeneration. Compared with Acvr1-only or Ilb1-only treatment, does the dual case show better regeneration?

- We thank the reviewer for this insightful comment. Our study was primarily designed to evaluate the effects of siACVR1 and siIL1b-mediated silencing on HO formation rather than to compare muscle regeneration among single- and dual-treatment groups. The restoration of muscle architecture observed in the dual-treatment group resulted from robust suppression of chondrogenic and osteogenic lesion formation. Because single-agent treatment with either siACVR1 or siIL1b was less effective at suppressing HO formation than dual silencing, muscle restoration in these groups would be expected to be correspondingly less pronounced. However, we cannot conclude that dual silencing promotes muscle regeneration to a greater extent than either siACVR1 or siIL1b alone, as comprehensive quantitative assessments of muscle regeneration were not performed across all treatment groups.

2. In Fig 3d, why did the authors measure Inhba (encoding Activin A), and how can we explain its upregulation in the FOP model compared with the control condition?

- We thank the reviewer for pointing this. We measured *Inhba* expression in FAP-lineage cells isolated from injured muscle of WT and FOP mice because *Inhba* expression provides a relevant mechanistic readout of the inflammatory and osteochondrogenic processes that drive HO formation. A previous study has reported that Activin A is rapidly induced in

activated immune cells during the early inflammatory response and promotes the production of pro-inflammatory cytokines, thereby initiating HO pathogenesis (PMID: 28986986). In addition to inflammatory cells, Activin A is highly expressed by Sox9+ mesenchymal and chondrogenic progenitors within developing HO lesions (PMID: 33563697). Furthermore, our previous study demonstrated that Activin A expression is markedly elevated in HO lesions following injury in FOP mice and that bone marrow-derived stromal cells (BMSCs) isolated from *Acvr1*^{R206H} mice exhibit heightened sensitivity to inflammatory stimuli, resulting in robust induction of *Inhba* expression (PMID: 37759764). Therefore, the increased *Inhba* expression in injured FOP tissues likely reflects contributions from both infiltrating inflammatory cells and HO-forming FAP-lineage cells, which establish a feed-forward pathogenic loop whereby inflammation induces Activin A production, and Activin A subsequently activates mutant ACVR1^{R206H} signaling to drive ectopic chondrogenesis and ossification.

3. In Fig 4, unconjugated siRNA accumulated in the kidney after 1 month of transdermal injection, but not in the liver. Compared with DCA conjugation, is this a general aspect of siRNA? The authors could mention the prevention of kidney accumulation as a benefit of DCA conjugation.

- We thank the reviewer for raising this point. This is a well-established phenomenon in the field. A previous study has demonstrated that unconjugated siRNAs (~13 kDa) fall below the renal filtration cutoff (~40 kDa) and are rapidly cleared via first-pass kidney filtration, with the retained fraction accumulating largely in kidney proximal tubule epithelial cells, a retention mechanism driven by the phosphorothioate backbone common to synthetic siRNAs (PMID: 30544191). In contrast, lipid conjugation alters serum protein binding and shifts this liver-to-kidney distribution ratio; DCA and other hydrophobic conjugates, in particular, reduce the contribution of renal filtration to overall clearance. Effectively, the conjugate dictates the kidney:liver distribution ratio. The kidney accumulation of unconjugated siRNA in our study, and its absence with DCA conjugation, is therefore consistent with this established mechanism. As this reflects a known, general property of siRNA conjugate pharmacology rather than a unique feature of our siRNA scaffold, we have not added it to the manuscript as a distinguishing benefit of DCA conjugation. Indeed, DCA-conjugated siRNA is still substantially retained in the liver, a primary clearance organ, so the improvement over unconjugated siRNA is better characterized as a shift in clearance route rather than avoidance of off-target tissue exposure.

4. As the authors briefly mentioned, transdermal routes may have a limitation when applied to large animals. They could discuss the feasibility and limitations of the transdermal approach using a microjet, based on their previous data, and clarify how this delivery strategy may translate to other models.

- We thank the reviewer for this insightful comment. Since intramuscular injection can induce tissue trauma that may trigger HO formation in FOP patients, we employed a hollow microneedle for transdermal (t.d.) delivery of siRNAs to minimize muscle injury. In addition, t.d. administered siRNAs exhibit efficient distribution and prolonged retention within skeletal muscle. Finally, hollow microneedle-based systems have been successfully used to deliver therapeutic agents into skin and underlying muscle tissues with minimal invasiveness. Thus, t.d. administration remains a feasible approach for localized treatment of injured muscle tissues and/or flare-up sites. However, we agree that the t.d. administration may present challenges when applied to larger animals or humans because of increased tissue thickness and the larger treatment areas that may require broader drug distribution. We have added a

discussion of these considerations and limitations to the revised discussion section (lines 633-636).

5. How do they differentially quantify wild-type and mutant Acvr1 using QRT-PCR?

- To differentially quantify wild-type and mutant *ACVR1*^{R206H} transcripts, we designed and screened multiple allele-specific qRT-PCR primer sets spanning the *ACVR1*^{R206H} mutation site (c.617G>A). We also included the primer sets previously used to detect the *ACVR1*^{R206H} mutation site, as reported in an earlier study (PMID:22508565). These primers were evaluated for their ability to selectively amplify either wild-type or mutant allele using mRNAs obtained from human wild-type bone marrow-derived stromal cells (BMSCs) and FOP patient-derived iPSCs. This optimized allele-specific qRT-PCR assay was subsequently used throughout the study to quantify the relative expression levels of wild-type and mutant *ACVR1*^{R206H} mRNA. We added this methodology into the revised method section (lines 817-824).

Reviewer #3 (Remarks to the Author):

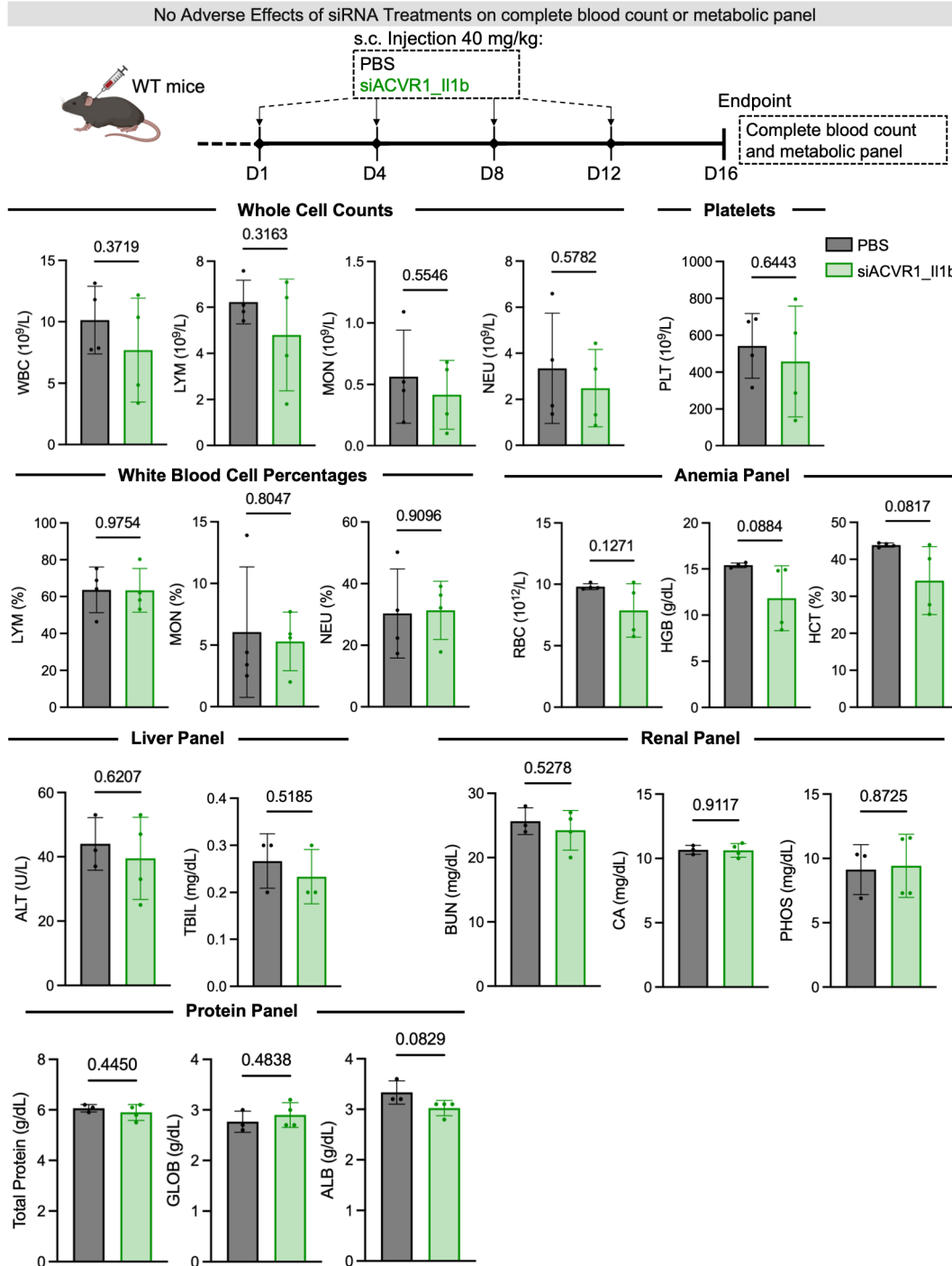
This manuscript describes an allele-selective siRNA (siACVR1) that discriminates the FOP-causing ACVR1R206H transcript from the wild-type allele, a second siRNA targeting IL1b/IL1B, and a unimolecular divalent construct (siACVR1_IL1b) combining both activities in a single DCA-lipid-conjugated molecule. The authors provide extensive in vitro screening, allele-selectivity validation in patient-derived iPSCs and ex vivo human muscle, in vivo efficacy in a trauma-induced heterotopic ossification (HO) mouse model via local and systemic delivery, mechanistic RNA-seq in FACS-sorted FAPs, and initial safety/toxicology data.

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- We thank the reviewer for the positive comments and for highlighting the significance of our manuscript. We appreciate the reviewer's constructive suggestions and believe that addressing these has strengthened the revised manuscript.

1) It seems the effectiveness of one dose can last 4-8 weeks, which are reasonably good for rodents. However, for clinical translation, repeatings are likely necessary. Therefore, it is important to identify toxicity and immunogenicity of repeat doses. Ideally, add a short repeat-dose pilot with corresponding toxicology to strengthen the safety package in Figure 6/Extended Fig. 18–19.

- We thank the reviewer for raising this important point. A key advantage of siRNA therapeutics is their capacity for durable gene silencing following infrequent dosing, which is well established clinically. GalNAc-conjugated siRNAs such as inclisiran achieve sustained target silencing with a twice-yearly maintenance dosing regimen (an initial dose, a second dose at 3 months, then every 6 months thereafter). Similarly, CBP-4888, a PC-DCA-conjugated siRNA targeting sFLT1 (PC-DCA being a positively charged, phosphocholine-modified analog of the DCA conjugate used in our study), is being developed by Comanche Biopharma as a single-dose therapy and is currently in a Phase 1 dose-escalation trial in hospitalized pregnant women with preterm preeclampsia (NCT07282171), having received FDA Fast Track designation. This precedent supports that durable, infrequent dosing is a favorable pharmacological property of lipid-conjugated siRNAs, including in highly sensitive patient populations, rather than a limitation requiring frequent re-dosing.
- Nonetheless, we agree with the reviewer's concern about potential toxicity and immunogenesis by repeat dosing of DCA-conjugated siRNAs. Thus, we performed a short-term repeat-dose tolerability study following subcutaneous administration of siACVR1_IL1b at 40 mg/kg every 4 days for 16 days (4 doses total). Complete blood counts and comprehensive metabolic panels were performed after the final dose, demonstrating little to no changes relative to PBS injections in any parameter, supporting the tolerability of repeat dosing. We have added these data to the revised manuscript (**Extended Fig. 20**).



2) The biodistribution data convincingly show the divalent scaffold improves muscle retention (Extended Fig. 11a). But the language around therapeutic efficacy implies the divalent molecule outperforms the monovalent combination, and I see no direct head-to-head statistical comparison between those two arms; each is compared separately against siNTC. Absent that comparison with appropriate correction, the Abstract and Discussion should frame the divalent construct's advantage as delivery, equimolar co-dosing, and manufacturability—compelling on their own—rather than therapeutic superiority.

- We agree with the reviewer's concern that direct statistical comparisons of therapeutic efficacy between the monovalent siRNA combination and the divalent siRNA were not included in the original analysis. We have now performed these head-to-head comparisons throughout the study and revised the Abstract and Discussion to specify that the advantage of the divalent siRNA scaffold lies in improved local tissue retention and duration, equimolar co-dosing, and manufacturability, rather than superior therapeutic efficacy, as suggested by the reviewer (lines 49-51, 595).
- Local (transdermal) delivery: The divalent siRNA scaffold shows significantly greater tissue accumulation and retention than the monovalent siRNA combination at both 1 and 3 months post-injection, consistent with improved local depot formation and supporting the durability advantage of this scaffold (**Fig. 4b**). However, direct comparison of *ACVR1^{R206H}* and *Il1b* silencing efficacy between the monovalent and divalent siRNA treatments showed little to no significant difference, both in bulk tissue ($p=0.8340$ and $p=0.7384$, respectively; **Extended Fig. 10a**) and in FACS-sorted FAPs and immune cells, the cell populations that drive HO pathology (**Extended Fig. 10b and c**).
- Systemic (subcutaneous) delivery: Direct comparison of *ACVR1^{R206H}* and *Il1b* silencing efficacy between the monovalent and divalent siRNA scaffolds in liver, kidney, and muscle, evaluated in both pre- and post-injury treatment paradigms, likewise showed little to no statistically significant differences (**Extended Fig. 16a and c**), consistent with comparable biodistribution between the two scaffolds (**Extended Fig. 15**).

3) *Is the treatment effect sex-dependent? Given known sex differences in BMP/activin signaling in some contexts, please report sex distribution and, if feasible, confirm findings are not sex-dependent.*

- We thank the reviewer for raising this point. We did not observe evidence of a sex-dependent treatment effect. Based on previous studies showing little to no sex-dependent differences in HO pathogenesis of FOP mice (PMID: 37759764, 36258013), both male and female mice were used in this study. WT and FOP mice were distributed across treatment groups without regard to sex, and we did not observe any apparent sex-specific differences in treatment outcomes. We have added this point to the revised manuscript (lines 751-754).

4) *Cy3-based "uptake" percentages (Extended Fig. 6, 11, 16) are conceptually distinct from per-cell knockdown magnitude; high Cy3+ percentages reflect internalization and don't guarantee productive cytoplasmic RISC loading in every cell type. For siRNA, one common challenge is the insufficient endosomal escape. It is important to determine the endosomal escape efficiency of the therapeutic siRNA, and measure their knockdown efficiency in a dose-dependent manner.*

- We agree with the reviewer's concern that Cy3-based uptake percentages reflect cellular internalization rather than productive cytosolic delivery. We would like to note, however, that direct, quantitative measurement of endosomal escape efficiency remains an unresolved technical challenge, not specific to our siRNA scaffolds. For example, while under 0.01% of internalized GalNAc-conjugated siRNA has been suggested to reach the cytosol (PMID: 32808038), no validated scalable assay currently exists to directly quantify this process for a therapeutic siRNA conjugate (PMID: 38437552). Given this limitation, we instead relied on the productive pharmaco-dynamic outcome, gene silencing itself, as the functional readout of sufficient endosomal escape and RISC loading.
- To address knockdown efficiency in a dose-dependent manner, we performed 7-point dose-response assays with siRNAs targeting mouse and human IL-1 β and human *ACVR1^{R206H}* in mouse and human cells and human muscle tissues (**Extended Fig. 2b-d, 9b and c, 21b-d**),

demonstrating potent, dose-dependent silencing efficiency. While this dose-response relationship does not directly quantify endosome-escaping siRNAs, it provides a broader functional indication that productive cytosolic delivery and RISC loading occurred in a dose-dependent manner, consistent with successful, if unquantified, endosomal escape.

5) Many supplementary panels present several pairwise comparisons (e.g., Extended Fig. 14c, three brackets per cell population; Extended Fig. 8a, 84 inflammatory genes screened simultaneously) using one-way ANOVA or unpaired t-tests, but I cannot find the post-hoc correction method stated. This matters most for the 84-gene screen underlying Il1b selection, where several hits cluster near $p \approx 0.02\text{--}0.05$ —precisely the range most sensitive to correction choice. Since Il1b as top hit is foundational to the second targeting arm, state the correction method and, ideally, report adjusted p-values for this screen.

- As suggested by the reviewer, we have applied the post-hoc corrections throughout the supplemental figures and indicated this in the corresponding figure legends. Additionally, we have now re-analyzed the RT2 Profiler PCR array data (**Extended Fig. 8a**) and added the sentence “Raw P values originally calculated for all 84 inflammatory genes were corrected for multiple testing using the Benjamini-Hochberg false-discovery-rate procedure” to the revised method section (lines 826-827). We have added the q values into our previous table. Il1b remained significant after correction, supporting the evidence of Il1b as the second targeting arm.

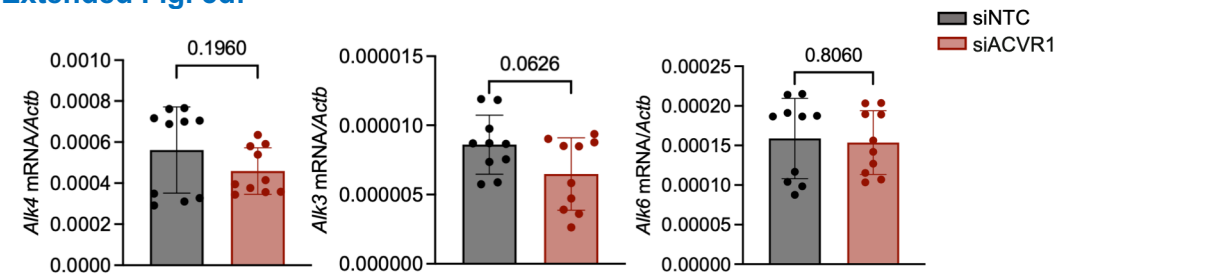
Position	Gene	fold change	p Value	q Value
A05	Ccl12	4.54	0.047385	0.159214
B01	Ccl4	2.78	0.031127	0.153804
B04	Ccl7	5.51	0.00518	0.134652
C04	Csf1	3.1	0.022442	0.134652
C09	Cxcl10	5.13	0.015512	0.118455
D09	Il10ra	4.22	0.046363	0.159348
E04	Il17b	2.57	0.038532	0.159348
E07	IL1b	9.1	0.000446	0.009366
E08	Il1r1	2.19	0.046101	0.159348
E09	IL1rn	4.73	0.018493	0.119493
G03	Pf4	5.1	0.024994	0.139966

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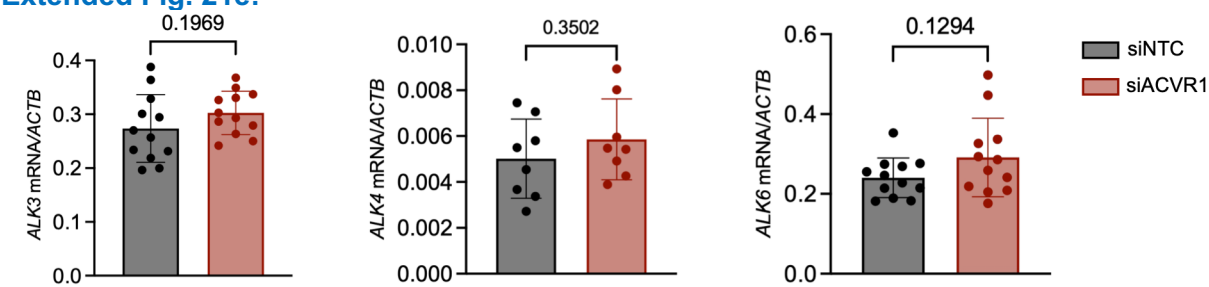
- As suggested by the reviewer, we measured the mRNA levels of the closely related paralogs ALK3, ALK4, ALK6, and ALK7 in mouse *Acv1^{R206H}* BMSCs (**Extended Fig. 5d**) and human muscle tissues (**Extended Fig. 21e**) following treatment with siACVR1. The expression levels of both mouse and human ALK3, ALK4, and ALK6, remained comparable between siNTC- and siACVR1-treated groups, indicating no detectable off-target silencing of these receptors. Notably, mRNA levels of ALK7 were below the limit of detection in both

mouse BMSCs and human muscle tissues. We have added these data to the revised manuscript (lines 194-196, 487-489).

Extended Fig. 5d:



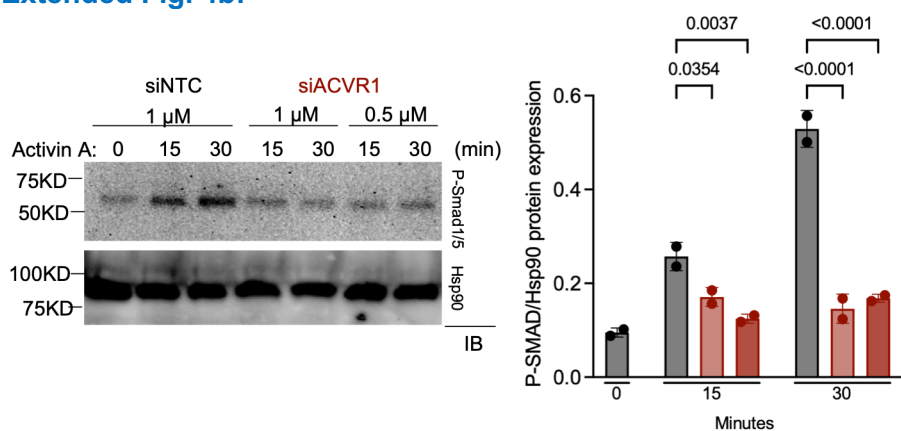
Extended Fig. 21e:



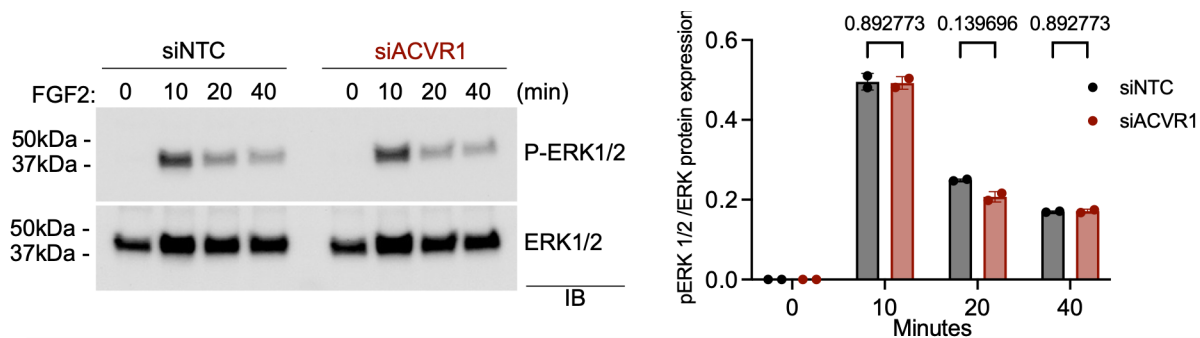
7) No densitometric quantification is provided for the P-SMAD1/5 (Extended Fig. 4b) or P-ERK1/2 (Extended Fig. 5c) immunoblots—only single representative lanes. Quantification across replicate blots, even n=2–3, would strengthen these claims beyond qualitative interpretation.

- As suggested by the reviewer, we performed densitometric quantification of P-SMAD1/5 or P-ERK1/2 immunoblots from two independent biological replicates and have added the quantification data to the revised manuscript (Extended Fig. 4b and 5c, right panels).

Extended Fig. 4b:



Extended Fig. 5c:



8) A detailed discussion on the design and selection of the allele-selective siRNA would be helpful for the readers to understand a general strategy in siRNA design. This is an important piece of value of this work.

- We thank the reviewer for this valuable suggestion. We have expanded the Discussion (SNP-selectivity section) to summarize our general strategy for designing allele-selective siRNAs (lines 531-543). Briefly, guide strands spanning the mutation site were generated and systematically screened by varying the position of a single primary mismatch to identify two key features: (1) the position that provided the greatest on-target efficacy and (2) the position that exhibited the greatest innate allele discrimination. After identifying lead positions with the highest levels of on-target mRNA knockdown and the strongest discrimination between wild-type and mutant mRNAs, a second screening step was performed in which a secondary mismatch was introduced into the flanking guide sequences to further enhance selectivity. This additional mismatch is necessary because a single mismatch alone is often insufficient to fully discriminate a single-nucleotide difference, given the RNAi machinery's tolerance for imperfect guide-target complementarity.
- From this secondary mismatch screen, candidates exhibiting the greatest target discrimination were selected and further evaluated in a seven-point dose-response assay to identify the lead sequence. Based on the results presented in this manuscript, together with our previous study that first described this strategy for therapeutic siRNAs (PMID: 36192390), we believe that this sequential screening approach is the most effective method for identifying SNP-selective siRNAs that function through a dual-mismatch mechanism. Notably, every siRNA evaluated in both this study and the original publication required a different combination of primary and secondary mismatch positions to achieve maximal target discrimination, suggesting that allele discrimination is highly sequence dependent. We therefore believe that this empirical dual-mismatch screening framework represents a broadly applicable strategy for the design of allele-selective siRNAs targeting other single-nucleotide disease variants, and we have highlighted this generalizability in the revised Discussion.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

- No reviewer comments were left for this revision.

REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

The authors substantially and appropriately responded to comments/suggestions from this reviewer and others.

: [Completed](#)

Reviewer #3 (Remarks to the Author):

The revised manuscript has adequately addressed my critiques/comments.

: [Completed](#)