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- 1 Supplementary information for:
- 2

## 3 Thermostable fatty acid hydroxylases from ancestral reconstruction of

#### 4 cytochrome P450 family 4 enzymes

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- 20 Supplementary Table 1. Substrate and hydroxylation regioselectivities of mammalian CYP4 enzymes towards FAs
- 21 Common FA substrates of the CYP4s are listed, along with the forms that metabolise them and regioselectivities if known (in the form of
- 22 terminal to subterminal i.e.,  $\omega$ : $\omega$ -1 ratio, unless otherwise stated).

FA Substrate	Form	Regioselectivity	Source		
Caprylic acid (C8)	Human CYP4B1 (S427P)	>1.5:1	1		
ОН	Rabbit CYP4B1	7.5:1	1		
Nonanoic acid (C9)	Human CYP4B1 (S427P)	1.6:1			
ОН	Rabbit CYP4B1	1.4:1	1		
Capric acid (C10)	Human CYP4B1 (S427P)	3.1:1			
ОН	Rabbit CYP4B1	1.1:1	1		
Lauric acid (C12)	Rat CYP4A1	40:1	2		

	Rat CYP4A8	25:1	
	Rat CYP4A2	>6:1	23
	Rat CYP4A3	>3:1	
	Rabbit CYP4A5	3:1	
	Rabbit CYP4A6	12:1	4
0	Rabbit CYP4A7	18:1	
ОН	Human CYP4A11	>15:1	2,5
	Mouse CYP4A10	16:1	
	Mouse CYP4A12a	5:1	6
	Mouse CYP4A12b	1:1.1	
	Mouse CYP4A14	1.6:1	
	Human CYP4B1 (S427P)	1.2:1	1

	Rabbit CYP4B1	3.5:1	
	Rat CYP4F4	ω	7
	Human CYP4Z1	ω-4>(2,3,5)	8
	Rat CYP4A1	3:1	
	Rat CYP4A2	3:1	
Myristic acid (C14)	Rat CYP4A3	1.2:1	2
ОН	Rat CYP4A8	1.6:1	
	Human CYP4A11	4:1	
	Human CYP4V2	ω-2>(3,4,5)	9
	Human CYP4Z1	ω-2	8
Palmitic acid (C16)	Rat CYP4A1	1:1	2
	Rat CYP4A8	1.6:1	

	Rabbit CYP4A4	4:1	
6			
	Rabbit CYP4A5	1.4:1	
			4
		2.7.1	
	Rabbit CYP4A6	2.7:1	
	Rabbit CVP/A7	2 5.1	
	Kabbit CTT4A7	2.3.1	
	Human CYP4A11	>2 2.1	2,5
		2.2.1	
	Rat CYP4A1	6:1	
	Rat CYP4A2	2.4:1	2
Arachidonic acid (C20:4)			
		2.1	
	Rat CYP4A3	2:1	
	Dabbit CVD4 A 4		
	Rabbit C I P4A4	ω	
	Rabbit CYP4A6	ω	4
	Rabbit CYP4A7	ω	
	Human CYP4A11	3.7:1	5

xMouse CYP4A10	4:1	
Mouse CYP4A12a	7:1	6
Mouse CYP4A12b	8:1	
Rabbit CYP4B1	ω-8	10-12
Rat CYP4F1	ω	7
Rat CYP4F4	ω	
Human CYP4F2	26:1	13,14
Human CYP4F3a	25:1	14
Human CYP4F3b	20:1	
Human CYP4F8	ω-2	15,16
Human CYP4F12	ω-2	16,17
Human CYP4X1	internal epoxidation	18

5-HETE			
ОН О ОН ОН ОН	Mouse CYP4A14	ω	19
8-HETE	Human CYP4F2	ω	20
НО, ОН	Mouse CYP4A14	ω	19
Prostaglandin H <sub>1</sub> O O O H O H	Human CYP4F8	ω-1>ω-2	15
Prostaglandin H <sub>2</sub>	Human CYP4F8	ω-1>ω-2	15



	Mouse CYP4A12b		
	Human CVP/F3a	2.1	
o o o o o o o o o o o o o o o o o o o	Tiuman C 11 41 Ja	2.1	14
ОН	Human CYP4F3b	1.7:1	
	Human CYP4V2	ω>ω-1	9
	Rat CYP4F1	ω	7
	Rat CYP4F4	ω	
Leukotriene B <sub>4</sub> (C20)	Rat CYP4F5	ω-2	21
HO,,,	Rat CYP4F6	ω-1, ω-2	21
он о	Human CYP4F2	ω	20
ОН	Human CYP4F3a	ω	22
	Human CYP4F3b	ω	22
	Mouse CYP4 <mark>F</mark> 14	ω	19

	Mouse CYP4F18	ω-1>ω-2	23
Lipoxin A <sub>4</sub>	Rat CYP4F1	ω	24
	Human CYP4F2	ω	20
НО Н	Mouse CYP4A14	ω	19
Docosapentaenoic acid (C22:5)			
ОН	Human CYP4F8 Human CYP4F12	(ω-2, ω-3)-epoxide	16
Docosahexaenoic acid (C22:6)	Human CYP4F3a	1.1:1	14
	Human CYP4F3b	2.1:1	14
	Human CYP4F12	(ω-2, ω-3)-epoxide	16
ОН	Human CYP4V2	ω-1>ω-2	9



#### 25 Supplementary Table 2. N-terminal modifications used for CYP4 expression

The known N-terminal modifications applied to extant members of the CYP4ABTXZ clade (hCYP4A11<sup>25</sup>, rCYP4B1<sup>26</sup>, hCYP4X1<sup>18</sup>) to facilitate recombinant expression in E. coli are compared with the corresponding native form of each sequence as well as the unmodified "native" sequences for each node of interest. The existing N-terminal modifications in the literature were used to help identify possible modifications to apply to each ancestor. Ultimately, the hCYP4A11-like modification was applied to CYP4ABTXZ, as the hCYP4A11 sequence showed the greatest sequence similarity (77%) to the ancestor. The rCYP4B1-like modification was applied to all intermediate nodes, as this sequence retained the greatest length of native sequence, allowing for subsequent shortening to apply alternative N-terminal modifications if required for expression.

Form	N-terminal sequence	Reference
hCYP4A11_Native	LLGDVSGILQAASLLILLLLIKAVQLYLHRQWLLKALQQFPCPPSHWLFGH	27
hCYP4A11_Modified	MALLLAVFLLLLIKAVQLYLHRQWLLKALQQFPCPPSHWLFGH	25
rCYP4B1_Native	MLGFLSRLGLWASGLILILGFLKLLRLLLRRQRLARAMDSFPGPPTHWLFGH	28
rCYP4B1_Modified	MALLLAVFGLWASGLILILGFLKLLRLLLRRQRLARAMDSFPGPPTHWLFGH	26
hCYP4X1_Native	LETRWARPFYLAFVFCLALGLLQAIKLYLRRQRLLRDLRPFPAPPTHWFLGH	29
hCYP4X1_Modified	MAKKTSSKGKL-PFPAPPTHWFLGH	18
CYP4ABTXZ_Native	LALLLLKAIQLYLRRQRLLRALQLFPGPPTHWLYGH	

CYP4ABTXZ_Modified (4A11-like)	MALLLAVFLALLLLKAIQLYLRRQRLLRALQLFPGPPTHWLYGH
CYP4ABTXZ_Modified (4B1-like)	MALLLAVFGLWASGLILALLLLKAIQLYLRRQRLLRALQLFPGPPTHWLYGH
CYP4ABTXZ_Modified (4X1-like)	MAKKTSSKGKLQLFPGPPTHWLYGH
CYP4B_Native	ETLVLLKAIQLYLRRQKLLKALESFPGPPTHWLYGH
CYP4B_Modified (4B1-like)	MALLLAVFGLWASGLILTLVLLKAIQLYLRRQKLLKALESFPGPPTHWLYGH
CYP4A_Native	LVLLLKAAQLYLRRQRLLRAFQSFPGPPAHWLYGH
CYP4A_Modified (4A11-like)	MALLLAVFLVLLLLKAAQLYLRRQRLLRAFQSFPGPPAHWLYGH
CYP4A_Modified (4B1-like)	MALLLAVFGLWASGLILVLLLLKAAQLYLRRQRLLRAFQSFPGPPAHWLYGH
CYP4XZ_Native	LALLLLQAIKLYLRRQRLLRALRLFPGPPTHWLYGH
CYP4XZ_Modified (4X1-like)	MAKKTSSKGKLRLFPGPPTHWLYGH
CYP4XZ_Modified (4B1-like)	MALLLAVFGLWASGLILALLLLQAIKLYLRRQRLLRALRLFPGPPTHWLYGH

Mass Transition
487>183.1
487>183
487>183
487>347
487>335
487>307
487>283
487>333
487>333
487>307
487>283
495>183.1
498>183.1
498>183.1
498>183.1

38 Supplementary Table 3. Mass transitions used to detect the AA metabolites and the internal

39 standards.

# 45 Supplementary Table 4. Estimated limit of detection (LOD) and limit of quantitation (LOQ)

	5,6-	8,9-	11,12-	14,15-	5-	9-	8-	12-	11-	15-	19-	20-
	ЕЕТ	EET	EET	EET	HETE							
LOD												
(fmol/mL;												
S/N = 3)	1597	349	127	57	343	179	61	167	121	45	89	43
LOQ												
(fmol/mL;												
S/N = 10)	5324	1164	423	190	545	598	202	558	405	150	297	144

46 for the measured EETs and HETEs

			20 30	40 5	0000 00000
	CYP4ABXZ CYP4B CYP4A CYP4X2 rCYP4B1 sCYP4T2 hCYP4A11 hCYP4X1 hCYP4Z1	MALLLAVFL MALLLAVFGLWASGLIL MALLLAVFGLWASGLIL MALLLAVFGLWASGLIL MELTEAFLTLHWGLPRLHHLLA MALLLAVF MEPSWLQELMAHPFLLLILLCM	ALLLIKAIQLYLRRQRLLR TLVLLKAIQLYLRRQKLLK VLLLLKAAQLYLRRQKLLK ALLLLQAIKLYLRQRLLR MAGFLKLLRLLLRRQRLAR LLCLVAVVYKLATLLAKR LLLLIKAVQLYLHRQWLLK SLLLFQVIRLYQRRRWMIR	A L Q L F PG P P T HWLY A L E S F PG P P T HWLY A L C S F PG P P A HWLY A L R L F PG P P T HWLY M D S F PG P P T HWLY D V F R S Y ED F PG P P T A L Q Q F P C P P S HWLF C K L P F P A P P T HWFL A L H L F P A P P A HWFY	HVREFQQEELQKF SHVHELQQEEELNKI SHVHELQQEEELNKI SHQREFQQEEELQKF SHALEIQKTGSLDKV HWLFGHVLEFKQDGT SHIQELQQDQELQRI SHKEFYP.VKEFEVY
			→ <u>0000000</u> 90 100	<u>2000000</u> 110 12	0 130
	CYP4ABXZ CYP4B CYP4A CYP4XZ rCYP4B1 sCYP4T2 hCYP4A11 hCYP4X1 hCYP4Z1	LEWVEKYPCAFPLWFGPFMVFL LSWAEKYPYAFPLWFGGFMAFL LSWAEKFPCAFPRWFSGFMVFL NELVEKYPCAFPLWVGPFQVFL VTWTQQFPYAHPLWVGQFIGFL DFDTLMAWTKQYPYAFPLWFGP QKWVETFPSACPHWLWGGKVRV EEIIEKYPRAFPFWIGPFQAFF HKLMEKYPCAVPLWVGPFTMFF	NIYDPDYAKVILSRRDPKS NIYHPDYAKAVFSRGDPKS QVYDPDYMKVILSRRDPKS NIYEPDYAKVLSRRDPKS NIYEPDYAKAVYSRGDPKA FFCVLNIHHPDYVKTILAS QLYDPDYMKVILGRSDPKS CIYDPDYAKILLKRQDPKS	HVSYKFLIPWIGKG NVSYKFLIPWIGKG QVSYKFLIPWIGKG PDVYDFFLQWIGKG TEPKDDVSYRFILS HGSYRFLAPWIGYG QYLQKFSPPLLGKG AVSHKILESWVGRG	LIVINGPKWFOHRRI LIVINGPKWFOHRRI LIVINGSTWFOHRRI LISIDGPKWFOHRRI LIVIDGPKWFOHRKI WIGEGILVSSGOKWF LILINGQTWFOHRRM LAALDGPKWFOHRRI LVTIDGSKWKKHRQI
		2 2022 202202020	• • • • • • • • • •	000000000000000000000000000000000000000	وو ووو
	CYP4ABXZ CYP4B CYP4A CYP4XZ rCYP4B1 sCYP4T2 hCYP4A11 hCYP4X1 hCYP4Z1	140 LTPGFHFSILKPYVALMADSVR LTPGFHYDILKPYVALMADSVR LTPGFHYDILKPYVALMADSVR LTPGFHYDILKPYVALMADSVR LTPGFHYDVLKPYVALMADSVR LTPGFHYDVLKPYVALFADSVR LTPAFHYDILKPYVGLMADSVR LTPGFHFNILKSYIEVMAHSVR VKPGFNISILKIFITMMSESVR	160 VMLDKWEKLIT.QDSSVEI VMLDKWEKLIT.QDSSVEI VMLDKWEKLIT.QDSSVEI VMLDKWEKLIT.QDSSVEI VMLDKWEKLIT.QDSSVEI IMLEKWEKLG.QDSPLEV VMLDKWEELLG.QDSPLEV MMLDKWEEHIA.QNSRLEI	TEONING THE TRANSLATION THE TR	CAFSHQTNCQTDSNS CAFSHQSNCQTDSDS CAFSHQSNCQTDSDS CAFSHQSNCQTDSTS CTFGKGDSGLNHRDS DSILKCAFSYNSNCQ CAFSHQGSIQVDRNS CAFSKETNCRTNSTH CAFSHQGSIQLDSTL
		.00000000000000000000000000000000000000		000000000000000000000000000000000000000	0000 0000
	CYP4ABXZ CYP4B CYP4A CYP4XZ rCYP4B1 sCYP4T2 hCYP4A11 hCYP4X1 hCYP4Z1	210 220 NSYIKAVYDLSYLVFHRLRNFL NSYIKAVYDLSYLVFHRLRNFL NSYIKAVYDLSYLVFSRIRNVF NSYIKAVFDLSKLIFHRLHNFL .SYYVAVSELTLLMQQRIDSFQ TESGTNVYIKAVYELSDLINLR QSYIQAISDLNNLVFSRVRNAF DPYAKAIFELSKIIFHRLYSLL DSYLKAVFNLSKISNQRMNNFL	230 240 YHNDLIYWFSPQGHRFRKA YHNDLIYWFSPQGHRFRKA HNDLIYWFSPQGHRFRKA HNDLIYFSPQGHRFQKI YHNDFIYWLTPHGRRFLRA LRTFPYHSDLIFYLSPHGY HONDTIYSLTSAGRWTHRA YHSDIIFKLSPQGYRFQKI HNDLVFKFSSQGQIFSKF	250 CQLAHQYTDKVIQE CQLAHQHTDKVIQE CQLAHQHTDQVIQK CQVAHQYTDKVIQE CRAAHDHTDRVIRQ RYRKAIKVAQSHTE CQLAHQHTDQVIQL SRVLNQYTDTIIQE NQELHQFTEKVIQ	260.270 RKESLKDEREQEKIQ RKESLKDERELEKIQ RKQHLQQEGELEKIQ RKASLQQEKEREKIQ RKASLQDEKEREKIQ VIKKRKEALKEEKE RKAQLQKEGELEKIK RKKSLQAGVKQDNTP RKESLKDKLKQTTQ
		000000 000	000000000000000000000000000000000000000	000000000000000000000000000000000000000	000000000000000000000000000000000000000
	CYP4ABXZ CYP4B CYP4A CYP4XZ rCYP4B1 sCYP4T2 hCYP4A11 hCYP4X1 hCYP4Z1	280 290 KKRHLDFLDILLCAKSENGEGL KKRHLDFLDILLCAKJENGEGL KKRYLDFLDILLCAKJENGDSL KKRYLDFLDILLCAKSENGEGL NRHLDFLDILLDVRGESGVQL LDRIQAKKNLDFLNILLLARDE RKRHLDFLDILLLAKMENGSIL KRKYQDFLDIVLSAKDESGSSF .KRRWDFLDILLSAKSENTKDF	300         310           SDEDLRAEVDTFMFEGHDT           SDEDLRAEVDTFMFEGHDT           SDEDLRAEVDTFMFEGHDT           SDEDLRAEVNTFMFEGHDT           SDEDLRAEVNTFMFEGHDT           SDEDLRAEVDTFMFEGHDT           SDEDLRAEVNTFMFAGHDT           SDEDLRAEVNTFMFEGHDT           SDEDLRAEVNTFMFEGHDT           SDEDLRAEVNTFMFEGHDT           SDEDLRAEVNTFMFEGHDT           SDKDLRAEVDTFMFEGHDT           SDKDLRAEVDTFMFEGHDT           SDIDVHSEVSTFLLAGHDT           SEADLQAEVKTFMFAGHDT	320 TASGISWLLYCMAM TASGISWLLYCMAL TASGISWLLYCMAM TASGISWLLYCMAM TSGISWFLYCMAM TTSGISWFLYCMAM TASGISWILYALAT LAASISWILYCLAL TSSAISWILYCLAK	330         340           NPEHQQRCREEIRGI           HPEHQQRCREEIRGI           HPEHQQRCREEIRGI           YPEHQQRCREEVRGI           YSLACHPEHQKICRD           HPKHQERCREEIRGI           YSLACHPEHQKICRD           HPKHQERCREEVRGI           YPEHQORCREEIRGL           YPEHQORCREEIRGL           YPEHQERCREEIRGL           YPEHQERCREEIRGL           YPEHQERCREEVRGI           YPEHQORCRDEIREL
		2 2222 222200		→ TT → TT ·	<u> </u>
	CYP4ABXZ CYP4B CYP4A CYP4XZ rCYP4B1 sCYP4T2 hCYP4A11 hCYP4X1 hCYP4Z1	LGDGDSITWDHLGCMPYTTMCI LGDGDSITWDHLGCMPYTTMCI LGDGASITWDHLGCMPYTTMCI LGDGDSITWDHLGCMPYTTMCI LGDQDSFQWEDLAKMTYLTMCM EIMQVLDGKDTMDWEDLSKIPY LGDGASITWNHLDCMPYTTMCI LGDGSSITWDQLGEMSYTTMCI LGDGSSITWEHLSCMPYTTMCI	KESLRLYPPVPSISRELSK KESLRLYPPVPSVSRQLSK KESLRLYPPVPSISRELSK KESLRLYPPVPSISRELSK KESLRLYPPVPSISRELSK KESLRLYPPVPOVYRQLSK KEALRLYPPVPGIGRELST KETCRLIPAVPSISRDLSK KECLRLYAPVVNISRLLDK	PITFPDGRSLPAGT PITFPDGRSLPAGT PITFPDGRSLPAGT PITFPDGRSLPAGM SKITKPITFCDGRT PVTFPDGRSLPKGII PUTFPDGRSLPKGII PUTFPDGRSLPKGII	IVVLNIYALHHNPTV LVSLNIYALHHNPTV IVSLNIYALHHNPRV IVVLNIWALHHNPTV LISLHIYALHRNSDV LPEGSYIGTSVFGIH MVLLSIYGLHHNPKV IVVLSIWGLHHNPAV TVFINIWALHHNPYF
		<u>ΤΤ 2222</u> Τ	T TT DOD DODD	0000000000000000000000	TT ->
	CYP4ABXZ CYP4B CYP4A CYP4XZ rCYP4B1 sCYP4T2 hCYP4A11 hCYP4X1 hCYP4Z1	WENPEVFDPLRESPENSSRRHS WEDPEVFDPLRESPENSSRRHS WEDPEVFDPLRESPENSSRRHS WENPQVFDPLRESPENSSGRH RNGIVWENPDVFDPLRESPENSSGRP WENPEVFDPLRESPENSSGRP WKNPKVFDPLRESPENSDQRHP WEDPQVFNPLRESRENSEKIHP	HAFLPFSAGPRNCIGQQFA HAFLPFSAGPRNCIGQQFA HAFLPFSAGSRNCIGQQFA HAFLPFSAGSRNCIGQQFA YAFIPFSAGPRNCIGQQFA SKRSPHAFVPFSAGPRNCI HAFLPFSGGSRNCIGKQFA YAYLPFSAGSRNCIGQEFA YAFIPFSAGLRNCIGQHFA	MIEMKVALALTLLR MNEMKVALALTLLR MNEMKVALALTLLR MNEMKVVIALALTLLR GONFAMNEMKVVIA MNELKVATALTLLR MIELKVTIALILLH IIECKVAVALTLLR	FELAPDPTKPPIPIP FELSPDPAKPPIPIP FELAPDPTKPPIPIP FELAPDPTKPPIPIP FEFSVDPLRIPIKL TLKKYHLIEDPNWK FELLPDPTRIPIPIA FRVTPDPTRPLTFPN FKLAPDHSRPPOPVR
48	CYP4ABXZ CYP4B CYP4A CYP4XZ rCYP4B1 sCYP4T2 hCYP4T1 hCYP4X1 hCYP4Z1	QIVLRSKNGIHLHLKKLH QIVLRSKNGIHLHLKKLQ QIVLRSKNGIHLHLKKLH QIVLRSKNGIHLHLKKLH QIVLRSKNGIHLHLKKLH QLVLRSKNGIHLYLKPLGPKAE PKIIPRLVLRSLNGIHLKKLSEC QVVLKSKNGIHVFAKKVC	510 		

#### 49 Supplementary Figure 1. Multiple sequence alignment of the CYP4 ancestors

#### 50 and corresponding extant forms.

51 The figure was created using ENDscript 3.0<sup>30</sup>. Red boxes indicate amino acid identity, red

52 characters indicate similarity, and blue frames surround regions of homology. The secondary

- 53 structure of rCYP4B1 is represented above the alignment in blue, with  $\alpha$ -helices depicted as
- 54 coils and  $\beta$ -sheets as arrows. Residue numbering is with respect to the numbering used for the
- 55 rCYP4B1 crystal structure (PDB: 5T6Q).





















9-HETE



5-HETE



19-HETE

- 58 Supplementary Figure 2. HETE formation by ancestral and extant CYP4s
- 59 Incubations of the ancestral and extant CYP4s with AA were carried out and metabolites
- 60 were analyzed with LC-MS. The data presented are the means +/- SD of three technical
- 61 replicates for all CYPs and six technical replicates for CYP-free controls (data points
- 62 collected on two separate days).



Supplementary Figure 3. EET formation by ancestral and extant CYP4s. 

Incubations of the ancestral and extant CYP4s with AA were carried out and metabolites were analyzed with LC-MS. The data presented are the means +/- SD of three technical replicates for all CYPs and six technical replicates for CYP-free controls (data points collected on two separate days).





75 standard showing chromatographic separation.



#### Supplementary Figure 5. AA oxidation by CYP4A11 (A), CYP4XZ (B), and 81

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CYP4ABTXZ (C). The data presented are the concentrations of 5-, 8-, 9-, 11-, 12-, 15-, 19-,
82
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HETE

HETE

HETE

HETE

HETE

- 83 and 20- HETE and 5,6-; 8,9-; 11,12-; and 14,15- EET from AA incubations in the presence of
- 84 NADPH or CuOOH. Asterisks indicate statistical significance compared to respective
- NADPH or CuOOH pCW control for each metabolite using an unpaired t-test (\*p < 0.05; \*\* 85
- $p \le 0.01$ , \*\*\* $p \le 0.001$ , \*\*\*\* $p \le 0.0001$ ). 86





11 HETE









89

19 HETE



12 HETE



9 HETE



5 HETE





96 one-tailed, t-test (\*p < 0.05; \*\* p  $\le$  0.01, \*\*\*p  $\le$  0.001, \*\*\*\*p  $\le$  0.0001).



98

99

Supplementary Figure 7. Stability of extant and ancestral P450s to incubation with CuOOH. Bacterial membrane preparations containing
0.6-1.0 µM of the P450s indicated were incubated with 500 µM CuOOH in 100 mM potassium phosphate buffer, pH 7.4. At the times indicated,

- 102 aliquots of incubations were removed and the residual P450 hemoprotein concentration was quantified by Fe(II).CO vs. Fe(II) difference
- 103 spectroscopy. Data represent the mean +/- SD of n=3 replicates.

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