Supplementary Information

Dias et al. 2019

Gene	Primers (5' > 3')	bp	Tm (ºC)	Amplicon Size	
OCT4	Fwd: GAGAACCGAGTGAGAGGCAACC	22	62.9	166	
0014	Rev: CATAGTCGCTGCTTGATCGCTTG	23	62.4	100	
NANOG	Fwd: AATACCTCAGCCTCCAGCAGATG	23	62.1	140	
	Rev: TGCGTCACACCATTGCTATTCTTC	24	61.9	149	
TCT5	Fwd: CACTGATAGGAACCCTAGAGGC	22	61.0	106	
FGF5	Rev: CAGATGGAAACCGATGCCC	19	60.5	190	
DAVC	Fwd: AGTGCCCGTCCATCTTTGC	19	62.6	01	
PAAO	Rev: CGCTTGGTATGTTATCGTTGGT	22	60.7	01	
P75	Fwd: ATCCTGGCTGCTGTGGTTGT	20	62.1	158	
	Rev: TCCACGGAGATGCCACTGTC	20	61.9		
т	Fwd: CTATTCTGACAACTCACCTGCAT	23	60.0	140	
1	Rev: ACAGGCTGGGGTACTGACT	19	61.9	140	
N 11 X/I 4	Fwd: TACCCCGACATCCACTTGCG	20	62.2	110	
MIALI	Rev: CCACTCTGACGCCGAGACTT	20	61.9	110	
MECD4	Fwd: CTGAAGGGCAGGCGATGGA	19	62.0	07	
MESPI	Rev: GGGCATCCAGGTCTCCAACA	20	61.9	03	
	Fwd: CCAAGGACCCTAGAGCCGAA	20	61.0	77	
INKA2.3	Rev: GTCCGCCTCTGTCTTCTCCA	20	61.3	11	
	Fwd: CACCTTCACCACCACCTCCC	20	62.4	207	
Ρυλι	Rev: CGTCCGCTTGTTCTCCTCCG	Rev: CGTCCGCTTGTTCTCCTCCG 20 63.1		207	
SOV17	Fwd: CTCCGGTGTGAATCTCCCC	19	59.5	04	
SUX17	Rev: CACGTCAGGATAGTTGCAGTAAT	23	58.6	74	
	Fwd: ACAACTTTGGTATCGTGGAAGG		60.2	101	
GAPDH	Rev: GCCATCACGCCACAGTTTC	19	61.7	101	

Melting temperatures (Tm), amplicon sizes and primer specificity were estimated using Primer Blast.



Figure S1. Cell morphology changes during cocktail exposure.

Condition with CHIR only shows a gradual change in cell morphology over exposure time, typically associated with a more differentiated-like phenotype, different of the morphology observed for controls and cocktails with TGF and/or FGF without CHIR, with more well-defined compact colonies, typically associated with the pluripotent state. Cell death was not increased when compared to controls, FGF only and TGF β only at any time point.



Figure S2. Full panel of the quadratic model for the pluripotency scores highlighting a dominant negative contribution of Wnt signaling.

7

Top, TGF β and FGF2 contributions to the pluripotency model with CHIR set to zero (A) and to $6 \mu M$ (B). Middle, FGF2 and CHIR contributions to the pluripotency model with TGF β set at zero (C) and at 2 ng/mL (D). Bottom, TGF\beta and CHIR contributions to the pluripotency model with FGF2 set at zero (E) and at 100 ng/mL (F). Overall, model shows a negative contribution of Wnt activation to pluripotency score. An FGF and CHIR synergy is also contributing to lower pluripotency scores.

	SS	df	MS	F	p-value
(1) FGF (L)	0.005	1	0.005	0.194	0.664
FGF (Q)	0.006	1	0.006	0.228	0.639
(2) TGF (L)	0.000	1	0.000	0.017	0.897
TGF (Q)	0.024	1	0.024	0.890	0.357
(3) CHIR (L)	6.010	1	6.010	219.264	6.9 x 10 ⁻¹²
CHIR (Q)	2.262	1	2.262	82.545	2.4 x 10 ⁻⁰⁸
1L by 2L	0.018	1	0.018	0.656	0.428
1L by 3L	0.148	1	0.148	5.402	0.031
2L by 3L	0.017	1	0.017	0.632	0.436
Error	0.521	19	0.027		
Total SS	8.013	28			

 Table S2. ANOVA Pluripotency score model.

SS (Sum of Squares); df (Degree of Freedom); MS (Mean of Squares); F (Fisher's Statistical test). In bold, statistically significant factors with p-values lower than 0.05.

	SS	df	MS	F	p-value
(1) FGF (L)	0.174	1	0.174	11.066	0.004
FGF (Q)	0.044	1	0.044	2.823	0.109
(2) TGF (L)	0.007	1	0.007	0.433	0.518
TGF (Q)	0.108	1	0.108	6.880	0.017
(3) CHIR (L)	3.466	1	3.466	219.985	6.7 x 10 ⁻¹²
CHIR (Q)	1.355	1	1.355	86.031	1.7 x 10 ⁻⁰⁸
1L by 2L	0.036	1	0.036	2.291	0.147
1L by 3L	0.070	1	0.070	4.425	0.049
2L by 3L	0.001	1	0.001	0.061	0.808
Error	0.299	19	0.016		
Total SS	6.242	28			

Table S3. ANOVA Ectoderm score model.

SS (Sum of Squares); df (Degree of Freedom); MS (Mean of Squares); F (Fisher's Statistical test). In bold, statistically significant factors with p-values lower than 0.05.



Figure S3. Full panel of the quadratic model for the ectoderm scores highlighting a dominant negative contribution of Wnt signaling with FGF signaling also contributing to lower ectoderm scores.

Top, TGF β and FGF2 contributions to the ectoderm model with CHIR set to zero (A) and to 6 μ M (B). Middle, FGF2 and CHIR contributions to the ectoderm model with TGF β set at zero (C) and at 2 ng/mL (D). Bottom, TGF β and CHIR contributions to the ectoderm model with FGF2 set at zero (E) and at 100 ng/mL (F). Overall, model shows a negative contribution of Wnt activation to ectoderm score. TGF and FGF signaling contributed negatively, while a synergy of CHIR and FGF contributed positively for ectoderm scores.



Figure S4. Full panel of the quadratic model for the mesendoderm scores highlighting a strong and dominant contribution of Wnt signaling.

Top, TGF β and FGF2 contributions to the mesendoderm model with CHIR set to zero (A) and to 6 μ M (B). Middle, FGF2 and CHIR contributions to the mesendoderm model with TGF β set at zero (C) and at 2 ng/mL (D). Bottom, TGF β and CHIR contributions to the mesendoderm model with FGF2 set at zero (E) and at 100 ng/mL (F). Overall, model shows a positive contribution of Wnt activation to mesendoderm score. Model predicts that CHIR 1 μ M contributes for negative mesendoderm scores.

	SS	df	MS	F	p-value
(1) FGF (L)	0.025	1	0.025	1.089	0.310
FGF (Q)	0.033	1	0.033	1.445	0.244
(2) TGF (L)	0.003	1	0.003	0.150	0.703
TGF (Q)	0.001	1	0.001	0.023	0.881
(3) CHIR (L)	11.565	1	11.565	500.279	4.1 x 10 ⁻¹⁵
CHIR (Q)	2.099	1	2.099	90.812	1.1 x 10 ⁻⁰⁸
1L by 2L	0.001	1	0.001	0.061	0.807
1L by 3L	0.063	1	0.063	2.711	0.116
2L by 3L	0.032	1	0.032	1.389	0.253
Error	0.439	19	0.023		
Total SS	13.627	28			

Table S4. ANOVA Mesendoderm score model.

SS (Sum of Squares); df (Degree of Freedom); MS (Mean of Squares); F (Fisher's Statistical test). In bold, statistically significant factors with p-values lower than 0.05

	SS	df	MS	F	p-value	
(1) FGF (L)	0.025	1	0.025	0.311	0.584	
FGF (Q)	0.034	1	0.034	0.418	0.526	
(2) TGF (L)	0.144	1	0.144	1.787	0.197	
TGF (Q)	0.094	1	0.094	1.166	0.294	
(3) CHIR (L)	11.764	1	11.764	145.963	2.3 x 10 ⁻¹⁰	
CHIR (Q)	10.879	1	10.879	134.987	4.5 x 10 ⁻¹⁰	
1L by 2L	0.001	1	0.001	0.013	0.911	
1L by 3L	0.320	1	0.320	3.971	0.061	
2L by 3L	0.060	1	0.060	0.744	0.399	
Error	1.531	19	0.081			
Total SS	22.548	28				

 Table S5. ANOVA Mesoderm score model.

SS (Sum of Squares); df (Degree of Freedom); MS (Mean of Squares); F (Fisher's Statistical test). In bold, statistically significant factors with p-values lower than 0.05.



Figure S5. Full panel of the quadratic model for the mesoderm scores highlighting the contribution of Wnt signaling with higher scores for intermediate CHIR concentrations.

Top, TGF β and FGF2 contributions to the Mesoderm model with CHIR set to zero (A) and to 6 μ M (B). Middle, FGF2 and CHIR contributions to the Mesoderm model with TGF β set at zero (C) and at 2 ng/mL (D). Bottom, TGF β and CHIR contributions to the endoderm model with FGF2 set at zero (E) and at 100 ng/mL (F). Overall, model shows a positive contribution of Wnt activation to mesoderm scores, with scores reaching their highest value at intermediate levels of activation. When FGF2 and

TGF β are full activated, higher concentrations of CHIR were needed to achieve the higher mesoderm score values (B).



Figure S6. Full panel of the quadratic model for the endoderm scores highlighted the contribution of Wnt signaling with FGF signaling also positively contributing to higher endoderm scores.

Top, TGF β and FGF2 contributions to the endoderm model with CHIR set to zero (A) and to 6 μ M (B). Middle, FGF2 and CHIR contributions to the endoderm model with TGF β set at zero (C) and

at 2 ng/mL (D). Bottom, TGF β and CHIR contributions to the endoderm model with FGF2 set at zero (E) and at 100 ng/mL (F). Overall, model shows a positive contribution of Wnt activation to endoderm score, with scores continuously increasing with CHIR concentration. FGF also provided a major contribution to higher endoderm scores.



Figure S7. Endoderm Model Score profiles and Standardized Effect Estimate. (A) CHIR endoderm profile shows an increase for all CHIR supplemented conditions in endoderm scores with CHIR concentration. The higher level of CHIR input alone contributed less to the endoderm score when compared to CHIR in combination with FGF or TGF. (B) CHIR quadratic and linear terms positively contributed to the endoderm score, with FGF2 linear term also contributing to higher endoderm scores. Model showed a good fit with a R² of 0.93 and a R²-Adjusted of 0.90.

	SS	df	MS	F	p-value
(1) FGF (L)	0.284	1	0.284	7.698	0.012
FGF (Q)	0.000	1	0.000	0.001	0.977
(2) TGF (L)	0.055	1	0.055	1.487	0.238
TGF (Q)	0.009	1	0.009	0.251	0.622
(3) CHIR (L)	8.044	1	8.044	218.185	7.2 x 10 ⁻¹²
CHIR (Q)	0.425	1	0.425	11.540	0.003
1L by 2L	0.006	1	0.006	0.154	0.700
1L by 3L	0.112	1	0.112	3.030	0.098
2L by 3L	0.000	1	0.000	0.001	0.977
Error	0.700	19	0.037		
Total SS	10.745	28			

Table S6. ANOVA Endoderm score model.

SS (Sum of Squares); df (Degree of Freedom); MS (Mean of Squares); F (Fisher's Statistical test). In bold, statistically significant factors with p-values lower than 0.05.