Supplemental material

Supplemental Table S1 : Comparison of metabolic profiles between ESR1 and ESR2 deficient patients

	ERα (ESR1)					ERβ (ESR2)
	Patient 1 *	**Patient 2 #**	**Patient 3 $**	**Patient 4 $**	**Patient 5 $**	**Our patient**
Age of presentation (years)	28	15	25	21	18	16.5
Karyotype	46, XY	46, XX	46, XX	46, XX	46, XY	46, XX
Gender identity	Male	Female	Female	Female	Male	Female
Insulin/HOMA		N	nd	nd	nd	N [HOMA 0.7 (<3.5)]
Chol tot		N	nd	nd	nd	↑ [5.4 mmol/l (<5.0)]
HDL		↑	nd	nd	nd	N [1.2 mmol/l (<1.0)]
LDL		N-↑	nd	nd	nd	↑ [3.8 mmol/l (<3.0)]
TGC		N	nd	nd	nd	N [0.8 mmol/l (<2.0)]

N: normal; nd: not done. * Ref 3; # Ref 4, $: Ref 5.

Supplemental Table S2: Amplification and sequencing primers and PCR length

	Sequence 5'-3'	***PCR bp***
primer Name; Exon		
ER2 exon 1-5'	CTCGGTCACGTGGGCTCAGGCAC	**490**
ER2 exon 1-3'	CTGGTTTCTCACCGCAGGATTTC	
ER2 exon 2-5'	CAGGATGTATTTGTAATCTCATAC	**626**
ER2 exon 2-3'	CCATAAAGTGATTTGAGAAATGGC	
ER2 exon 3-5'	GCTTTGCTGTATCAGATTTCCGG	**447**
ER2 exon 3-3'	GCTCATCAAATACTTTGTGTGCC	
ER2 exon 4-5'	GTACCTGTACTGGTCATTAAGAAG	**390**
ER2 exon 4-3'	GCCCCCTCCAATGTGACAACACG	
ER2 exon 5-5'	CGACCATAGTAATCTTTGGTACG	**503**
ER2 exon 5-3'	GGACCTCTACTTTGTACTCTTGC	

ER2 exon 6-5'	GGGTCCAAGACTGGAAACTAAG	**421**
ER2 exon 6-3'	CAGCACCCAGGACTTTGTTCCC	
ER2 exon 7-5'	GATATTATCAGATGAACATGTTAC	**333**
ER2 exon 7-3'	CATTCTTCTTAATATCACGCTAG	
ER2 exon 8-5'	GTCAAAGCAACCCAGATCACCTC	**390**
ER2 exon 8-3'	CATTTTCCTTAACTTGCAGACAC	
ER2 exon 9-5'	GGTAGACTGGCTCTGAGCAAAG	**399**
ER2 exon 9-3'	CTGCCATCACCAAATGAGGGAC	

Primer for Exon 5	***Sequence 5'-3'***
Exon 5 forw	GCCGACAAGGAGTTGGTACACA
Exon 5 rev	TCCAGAATTCCTTCTACGCAT

Supplemental Table S3: **<u>Site –directed mutagenesis primers</u>**

site directed Mutagenesis: cDNA. 1409A >C

Primer	**Sequence**	**AnnealingTemp °C**
ERb mt frw	GATCAGCTGGGCCAGGAAGATTCCCGGCT	**78**
Erb mt rev	AGCCGGGAATCTTCCTGGCCCAGCTGATC	**78**

Supplemental Table S4

Variants found in genes which are known to be implicated in 46,XY and 46,XX DSD [ref. Baetens et al.]. The occurrence in the general population and in the African population (AF) was extracted from gnomAD (rare variants: MAF<0.01). The pathogenicity of missense variants was calculated using PANTHER, PhD-SNP, SNAP, Meta-SNP, SIFT and PolyPhen. Scores representing predictions of damaging effects were colored in red.

Baetens, D., et al., *NR5A1 is a novel disease gene for 46,XX testicular and ovotesticular disorder of sex development*. Genet Med, 2017. 19:367-376.

In the methods, we need to change from ExAC to gnomAD (Genome Aggregation Database, [M., Lek, et al., Analysis of protein-coding genetic variation in 60’706 humans. Nature 536, 2016].

Genes	Zygosity	AA substitution	rs ID	gnomAD	gnomAD (AF)	PANTHER	PhD-SNP	SNAP	Meta-SNP	SIFT	PolyPhen
AKR1C1	Het	K39R	rs149693250	0.00118	0.01256	0.549	0.305	0.13	0.59	0.35	0.363
AKR1C2	Hom	F46Y	rs2854482	0.04039	0.1536	0.505	0.125	0.345	0.188	1	0.038
AKR1C4	Het	G135E	rs11253043	0.008507	0.08816	---	0.783	0.63	0.565	0.18	0.55
AKR1C4	Hom	R250Q	rs4880718	0.9999	0.9997	0.3	0.547	0.665	0.515	0.03	0.001
AMH	Het	S49I	rs10407022	0.764	0.5694	---	0.206	0.625	0.137	0	0.068
AMH	Het	P317R	rs566806768	0.0003694	0.004207	---	0.255	0.565	0.111	0.06	0.81
AMH	Hom	A515V	rs10417628	0.9817	0.9966	---	0.209	0.575	0.127	0.15	0
ATRX	Hom	E929Q	rs3088074	0.3843	0.8772	---	0.105	0.635	0.13	0	0
CYP21A2	Het	S203G	rs372964292	0.0001836	0.001943	0.102	0.09	0.155	0.303	0.39	0.002
FSHR	Het	A307T	rs6165	0.5459	0.2746	0.058	0.119	0.53	0.192	0.4	0
FSHR	Het	S680N	rs6166	0.5739	0.5881	---	0.16	0.49	0.134	0.49	0.005
GATA4	Het	V380M	rs114868912	0.005675	0.03339	0.106	0.236	0.41	0.266	0.23	0.001
HSD17B1	Het	G313S	rs605059	0.5529	0.5007	---	0.226	0.685	0.097	0.02	0.002
HSD3B2	Het	L236S	rs35887327	0.003788	0.0399	0.357	0.612	0.18	0.448	0.11	0.034
KAL1	Hom	V534I	rs808119	0.5578	0.3548	---	0.368	0.525	0.245	1	0.001
KISS1R	Het	L364H	rs350132	0.7941	0.897	---	0.251	0.375	0.174	0.57	0
LEPR	Het	K656N	rs1805094	0.1586	0.1944	0.336	0.168	0.57	0.161	0.17	0.106
MAMLD1	Het	V480A	rs61740566	0.01759	0.1764	0.096	0.019	0.575	0.091	0.01	0
MAMLD1	Het	Q807R	---	---	---	---	0.041	0.48	0.126	0.07	0.402
MAP3K1	Het	A671V	rs201310278	0.0001809	0.000833	---	0.49	0.365	0.373	0.07	0.122
MAP3K1	Het	D806N	rs702689	0.5963	0.4899	---	0.396	0.555	0.112	0.19	0.092
MAP3K1	Het	V906I	rs832582	0.7603	0.7467	---	0.137	0.51	0.059	0.76	0
NELF	Het	A64V	---	---	---	---	0.128	0.585	0.069	0.1	0.028
RSPO1	Het	K162Q	rs36043533	0.04992	0.009377	---	0.245	0.52	0.189	0.01	0.259
WWOX	Het	L216V	rs7201683	0.01883	0.1004	0.206	0.177	0.525	0.298	0.31	0.012
ZFPM2	Het	A403G	rs11993776	0.1169	0.3925	0.207	0.016	0.37	0.1	1	0

Supplemental Table S5 : Variants in genes known to be involved in Premature Ovarian Failure (POF)

Genes	**Zygosity**	**Variant**	**rs ID**	**gnomAD**	**gnomAD (AF)**	**PANTHER**	**PhD-SNP**	**SNAP**	**Meta-SNP**	**SIFT**	**PolyPhen**
DIAPH2	Het	Intron variant: c.104+61T>C	rs20386	0.2124	0.1846	---	---	---	---	---	---
	Het	Intron variant: c.165+94A>G	rs5949989	0.2215	0.2606	---	---	---	---	---	---
	Het	Intron variant: c.447+4796T>A	rs11339405	0.00032	0	---	---	---	---	---	---
	Het	Intron variant: c.587+2689G>A	rs2642219	0.3334	0.6511	---	---	---	---	---	---
	Het	Intron variant: c.587+2742T>C	rs2642218	0.3354	0.6597	---	---	---	---	---	---
	Het	Intron variant: c.733-20delT	rs756976277	0.3035	0.3024	---	---	---	---	---	---
	Hom	Intron variant: c.1444+40G>A	rs5920973	0.9972	0.9727	---	---	---	---	---	---
	Het	Intron variant: c.2349+69A>G	rs5920744	0.1147	0.2971	---	---	---	---	---	---
	Hom	3'-UTR variant: c.*368dupA	rs79669248	0.3428	0.4356	---	---	---	---	---	---
	Hom	3'-UTR variant: c.*1431_1433dupATA	rs867708140	0.3368	0.4204	---	---	---	---	---	---

	Hom	3'-UTR variant: c.*3841_3842insTA	rs10667776	0.8889	0.8132	---	---	---	---	---	---
ERCC6	Hom	Intron variant: c.1821+7C>T	rs4253132	0.8895	0.7235	---	---	---	---	---	---
	Hom	Intron variant: c.1992+32A>G	rs4253162	0.9076	0.729	---	---	---	---	---	---
	Het	Synonymous variant: G917G	rs2229760	0.3372	0.1312	---	---	---	---	---	---
	Het	M1097V	rs2228526	0.2161	0.1302	---	0.125	0.67	0.122	0.02	0.003
	Het	R1213G	rs2228527	0.2179	0.1457	---	0.545	0.74	0.465	0	0.13
	Het	Q1413R	rs2228529	0.2155	0.1244	---	0.088	0.605	0.115	0.2	0.015
	Het	T1441I	rs4253230	0.006874	0.0724	---	0.542	0.65	0.415	0.02	0
	Het	3'-UTR variant: c.*379C>G	rs4253234	0.3173	0.1345	---	---	---	---	---	---
FIGLA	Het	Intron variant: c.231+24T>C	rs12713717	0.3422	0.3428	---	---	---	---	---	---
	Hom	S141T	rs7566476	0.6288	0.8242	---	0.15	0.44	0.156	1	0
	Hom	Synonymous variant: H184H	rs7566541	0.6539	0.9132	---	---	---	---	---	---
	Het	3'-UTR variant: c.*5T>A	rs56135050	0.273	0.05603	---	---	---	---	---	---
	Het	3'-UTR variant: c.*7A>G	rs56316086	0.2729	0.05619	---	---	---	---	---	---

FMR1	Hom	Intron variant: c.104+61T>C	rs1270092	1	1	---	---	---	---	---	---
	Het	Synonymous variant: R138R	rs26707	0.08252	0.1737	---	---	---	---	---	---
	Hom	5'-UTR variant: c.-155C>T	rs4824233	1	1	---	---	---	---	---	---
FOXL2	Het	3'-UTR variant: c.*dupA	rs555195919	0.0999	0.2376	---	---	---	---	---	---
HFM1	Het	5'-UTR variant: c.-83C>G	rs17131429	0.2739	0.08447	---	---	---	---	---	---
	Hom	V117I	rs282009	0.9996	0.9962	---	0.19	0.425	0.059	1	0
	Hom	Synonymous variant: Q211Q	rs282026	0.9996	0.9962	---	---	---	---	---	---
	Hom	Intron variant: c.873+95G>T	rs282033	0.9987	0.9954	---	---	---	---	---	---
	Hom	Synonymous variant: I1033I	rs281992	0.7044	0.7422	---	---	---	---	---	---
	Hom	Synonymous variant: A1357A	rs585898	0.9996	0.9962	---	---	---	---	---	---
MCM8	Hom	Intron variant: c.336+49T>C	rs236114	0.836	0.9154	---	---	---	---	---	---
	Hom	Intron variant: c.1223+35A>G	rs236100	1	1	---	---	---	---	---	---

MSH5	Het	5'-UTR variant: c.-787T>A	rs707915	0.0827	0.2078	---	---	---	---	---	---
	Het	Synonymous variant: Q733Q	rs707938	0.3868	0.6491	---	---	---	---	---	---
NOBOX	Hom	Synonymous variant: L88L	---	4.068E-06	0	---	---	---	---	---	---
	Hom	Intron variant: c.292+66A>G	rs727717	9.348E-06	0.0001736	---	---	---	---	---	---
	Hom	Intron variant: c.1154+11T>C	rs757388	0.6294	0.6462	---	---	---	---	---	---
	Hom	Intron variant: c.1155-23G>A	rs11769847	0.6335	0.6466	---	---	---	---	---	---
	Hom	Intron variant: c.1240+34G>A	rs11769787	0.4686	0.4272	---	---	---	---	---	---
	Hom	Intron variant: c.1469+163A>G	rs2699502	0.5801	0.6174	---	---	---	---	---	---
	Hom	F517L	rs2699503	0.6241	0.6264	---	0.182	0.705	0.222	0	0.619
SF1/NR5A1	Het	Intron variant: c.871-20C>T	rs2297605	0.4806	0.2212	---	---	---	---	---	---
	Het	3'-UTR variant: c.*82C>T	rs915043	0.01182	0.03986	---	---	---	---	---	---

	Het	3'-UTR variant: c.*173delG	rs5900617	0.5552	0.7207	---	---	---	---	---	---
	Het	3'-UTR variant: c.*903C>T	rs10120967	0.5649	0.7501	---	---	---	---	---	---
	Het	3'-UTR variant: c.*1400T>C	rs10283445	0.5641	0.7444	---	---	---	---	---	---
STAG3	Hom	L16F	rs11531577	0.1803	0.05636	---	0.121	0.365	0.057	0.11	0
	Hom	T36P	rs2272343	0.1812	0.06552	0.579	0.567	0.225	0.478	0.29	0
	Hom	Intron variant: c.220-63G>A	rs2056726	0.1632	0.05714	---	---	---	---	---	---
	Hom	Synonymous variant: P431P	rs3735241	0.5079	0.3905	---	---	---	---	---	---
SYCE1	Hom	5'-UTR variant: c.-110G>G	rs3020503	0.8161	0.3358	---	---	---	---	---	---
	Hom	Intron variant: c.528+203C>G	rs2987791	0.7104	0.2733	---	---	---	---	---	---
	Hom	Intron variant: c.830+47T>C	rs3020514	0.9671	0.8474	---	---	---	---	---	---

Variants found in genes which are implicated in premature ovarian failure (POF). The occurrence in the general population and in the African population (AF) was extracted from gnomAD (rare variants: MAF<0.01). The pathogenicity of missense variants was calculated using PANTHER, PhD-SNP, SNAP, Meta-SNP, SIFT and PolyPhen. Scores representing predictions of damaging effects were colored in red.

Supplemental Table S6

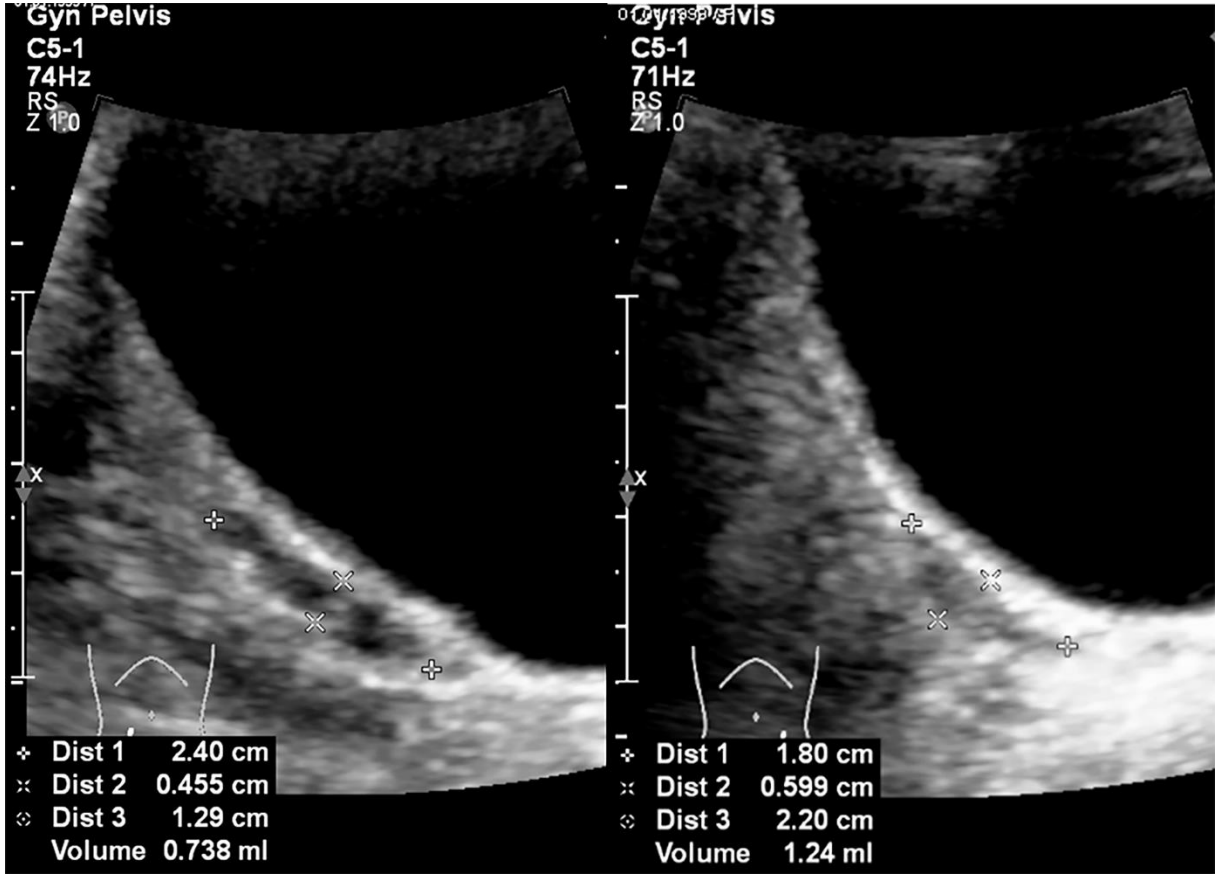
Table Pathway Studio:

Variants found in genes up- and downstream of ESR2. The occurrence in the general population and in the African population (AF) was extracted from gnomAD (rare variants: MAF<0.01). The pathogenicity of missense variants was calculated using PANTHER, PhD-SNP, SNAP, Meta-SNP, SIFT and PolyPhen. Scores representing predictions of damaging effects were colored in red.

	Gene	Zygosity	Variant	rs ID	gnomAD	gnomAD (AF)	PANTHER	PhD-SNP	SNAP	Meta-SNP	SIFT	PolyPhen
	ESR2	Het	K314R	---	0	0	0.826	0.664	0.42	0.541	0.03	0.999
Upstream of ESR2	**EYA2**	Hom	Premature start codon: c.-329G>C	rs13043776	1	1	---	---	---	---	---	---
	NCOR2	Het	S1508T	rs75902515	0.003231	0.02556	0.162	0.043	0.365	0.067	0.25	0.202
	MSR1	Het	Premature start codon: c.-163G>A	rs188206282	0.00003257	0.0001158	---	---	---	---	---	---
	CAV3	Het	T78M	rs72546668	0.002674	0.004041	0.457	0.282	0.58	0.326	0.06	0.537
	NCOA3	Het	H327Y	rs780175917	0.000002887	0	0.219	0.566	0.585	0.707	0.13	0.02
Up- and Downstream of ESR2	**ESR1**	Het	5'-UTR variant: c.-73A>G	rs867240	0.03156	0.1085	---	---	---	---	-	-
	EGFR	Het	Synonymous variant: G544G	rs17290103	0.002698	0.02808	---	---	---	---	-	-
	EGFR	Het	Intron variant: c.241-8C>G	rs138872748	0.0004292	0.00008321	---	---	---	---	-	-

Downstream of ESR2	**PTGS1**	Het	W8S	rs142176470	0.0004247	0.004466	0.071	0.115	0.51	0.08	0.46	0
	PTGDS	Het	Intron variant: c.115-9T>C	rs35556170	0.009784	0.1017	---	---	---	---	---	---
	PTGDS	Het	Intron variant: c.115-8C>T	rs34184410	0.009795	0.1017	---	---	---	---	---	---
	DRD2	Het	S311C	rs1801028	0.02576	0.003748	---	0.247	0.48	0.184	0.21	0.999

Supplemental Figure S1

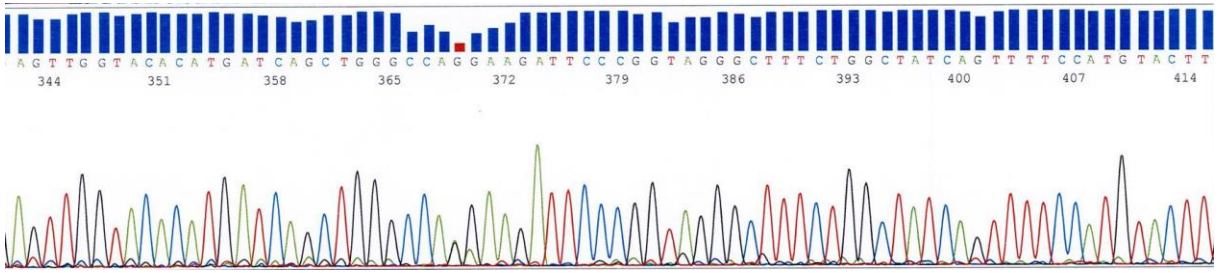


Abdominal ultrasound

Ultrasonography under estrogen replacement therapy revealed streak ovaries without follicles (right ovary left panel; left ovary right panel).

Supplemental Figure S2

A.

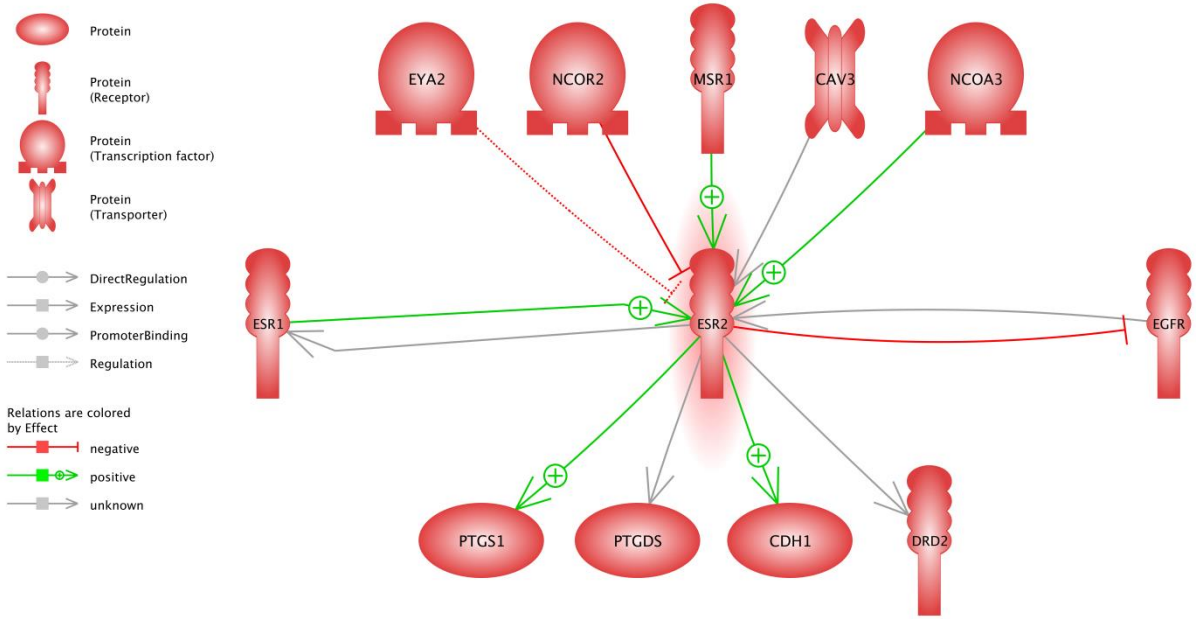


B.

```
Human            279  HVL-ISRPSAPFTEASMMMSLTKLADKELVHMISWAKKIPGFVELSLFDQVRLLESCWMEVLMMGLMWRSIDHPGKLIFA  357
Pigmy chimpanzee 279  HVL-ISRPSAPFTEASMMMSLTKLADKELVHMISWAKKIPGFVELSLFDQVRLLESCWMEVLMVGLMWRSIDHPGKLIFA  357
Gorilla          279  HVL-ISRPSAPFTEASMMMSLTKLADKELVHMISWAKKIPGFVELSLFDQVRLLESCWMEVLMVGLMWRSIDHPGKLIFA  357
Macaque          279  HVL-ISRPSAPFTEASMMMSLTKLADKELVHMISWAKKIPGFVELSLFDQVRLLESCWMEVLMVGLMWRSIDHPGKLIFA  357
Orangutan        280  HVL-ISRPSAPFTEASMMMSLTKLADKELVHMISWAKKIPGFVELSLFDQVRLLESCWMEVLMVGLMWRSIDHPGKLIFA  358
Olive baboon     279  HVL-ISRPSAPFTEASMMMSLTKLADKELVHMISWAKKIPGFVELSLFDQVRLLESCWMEVLMVGLMWRSIDHPGKLIFA  357
Spider monkey    280  HVL-ISRPSVPFTEASMMMSLTKLADEELVHMISWAKKIPGFVELSLLDQVRLLESCWLEVLMVGLMWRSIDHPGKLIFA  358
Mouse            298  NVL-VSRPSMPFTEASMMMSLTKLADKELVHMIGWAKKIPGFVELSLLDQVRLLESCWMEVLMVGLMWRSIDHPGKLIFA  376
Rat              234  NVL-VSRPSMPFTEASMMMSLTKLADKELVHMIGWAKKIPGFVELSLLDQVRLLESCWMEVLMVGLMWRSIDHPGKLIFA  312
Bovine           276  HVL-ISRPSTPFTEASMMMSLTKLADKELVHMISWAKKIPGFVELSLYDQVRLLESCWLEVLMVGLMWRSIDHPGKLIFA  354
Swine            276  HVL-VSRPSTPFTEASMMMSLTKLADKELVHMISWAKKIPGFMELSLYDQVRLLESCWLEVLMVGLMWRSIDHPGKLIFA  354
Sheep            276  HVL-MSRPSAPFTEASMMMSLTKLADKELVHMISWAKKIPGFVELSLYDQVRLLESCWLEVLMVGLMWRSIDHPGKLIFA  354
Goat             276  HVL-MSRPSAPFTEASMMMSLTKLADKELVHMISWAKKIPGFVELSLYDQVRLLESCWLEVLMVGLMWRSIDHPGKLIFA  354
Horse            299  NVL-VSRPSTPFTESSMMMSLTKLADMELVHMIGWAKKIPGFVELSLFDQVRLLESCWLEVLMVGLIWRSIDHPGKLIFA  377
Rhinocerus       298  HVL-VSRPSSPFTESSMMMSLTKLADMELVHMIGWAKKIPGFVELSLFDQVRLLEGCWLEVLMVGLIWRSIDHPGKLIFA  376
Chicken          232  NVL-VSRPSKPFTEASMMMSLTKLADKELVHMIGWAKKIPGFIDLSLYDQVRLLESCWMEVLMIGLMWRSIDHPGKLIFA  310
Star             304  HVL-VSRPSKPFTEASMMMSLTKLADKELVHMIGWAKKIPGFIDLSLYDQVRLLESCWMEVLMIGLMWRSIDHPGKLIFA  382
Alligator        304  NVL-VSRPNKPFTEASMMMSLTKLADKELVHMIGWAKKIPGFIELSLYDQVRLLESCWMEVLMVGLMWRSIDHPGKLIFA  382
Salmon           313  EIYlQKDMRRPLTEANVMMSLTNLADKELVHMISWAKKIPGFVDLCLFDQVHLLECCWLEVLMLGLMWRSVGHPGRLIFS  392
Dogfish          300  NVYsLNHPNKPYTEVSMMMSLTNLADRELVHMIAWAKKVPGFVELDLHDQVQLLECCWLEVLMVGLMWRSIEYPGKLLFA  379
Goldfish         307  EIYlMKDVKKPFTEANVMMSLTNLADKELVHMISWAKKIPGFVEIGLFDQVHLLECCWLEVLMLGLMWRSVNHPGKLVFS  386
Zebrafish        311  QIYlREPVKKPYTEASMMMSLTSLADKELVLMISWAKKIPGFVELTLSDQVHLLECCWLDILMLGLMWRSVDHPGKLIFT  390
```

Targeted sequencing of the ESR2 mutation identified by whole exome sequencing in the patient. Representative electropherograms of the sequencing performed in the patient's DNA showing the location of the heterozygote mutation (arrow) (Panel A). Alignment of the substituted Lysine 314 with other species shows complete conservation in all 22 species (Panel B)

Supplemental Figure S3

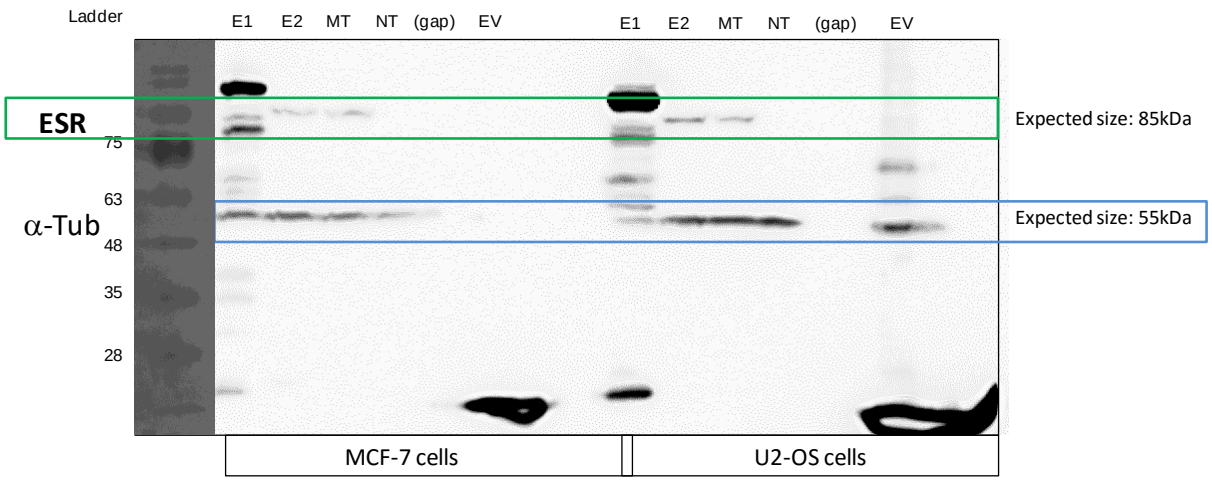


Mutated genes upstream of ESR2: EYA2 (EYA Transcriptional Coactivator and Phosphatase 2), EGFR (Epidermal Growth Factor Receptor), NCOR2 (Nuclear Receptor Corepressor 2), MSR1 (Macrophage Scavenger Receptor 1), CAV3 (Caveolin 3), ESR1 (Estrogen Receptor 1) and NCOA3 (Nuclear Receptor Coactivator 3).

Mutated genes downstream of ESR2: ESR1, EGFR, PTGS1 (Prostaglandin-Endoperoxide Synthase 1), PTGDS (Prostaglandin D2 Synthase), CDH1 (Cadherin 1) and DRD2 (dopamine Receptor 2)

Supplemental Fig. S4

Western blot analysis of protein extracts from MCF7 and U2-OS cells transfected with ESR1 (E1), ESR2 wild type (E2) and mutant (MT) demonstrating that the differences in transactivation activity are not due to differences in protein quantity or stability.



Supplemental Fig.S5