SUPPLEMENTAL APPENDIX: A RECURRENT *CYP3A4* MUTATION AS A NOVEL CAUSE OF RICKETS

Supplementary Methods **2**

Isolation of genomic DNA and Sanger sequencing....2

In Vitro assessment of expression and activity of CYP3A4....2

Semi-quantitative immunoblot of transfected CYP3A4 protein....2

Determination of uncommonly used reference intervals....2

More detailed information regarding the clinical course of each subject....2

Subject 1.1....2

Subject 2.1....2

Supplementary Tables **3**

Table S1. Rare variants identified in subject 1.1 & subject 2.1 that fit an autosomal dominant model of disease....3

References **8**

Supplementary Methods

Public databases searched for rare variants

1,000 Genomes Project, ESP6500SI, ExAC, and gnomAD

Cell lines

All cell lines were obtained from ATCC and have been cultured continuously in our lab.

Isolation of genomic DNA and Sanger sequencing

We extracted genomic DNA from peripheral blood mononuclear cells of affected patients and available family members using standard methods. Candidate genes were analyzed by Sanger sequencing of both strands.

In Vitro assessment of expression and activity of CYP3A4

Mutations were introduced by PCR site-directed mutagenesis into a full-length human *CYP3A4* cDNA (generously provided by Dr. P.F. Hollenberg, University of Michigan, Ann Arbor, Michigan, USA(1)), and nucleotide changes were verified by sequencing. Expression of recombinant CYP3A4 proteins was assessed by immunoblot analysis of whole cell lysates of transiently transfected HEK293T cells. We used a mammalian two-hybrid expression system in which activity of a firefly luciferase reporter protein is proportional to the concentration of intracellular 1,25-dihydroxyvitamin D to assess the ability of recombinant CYP enzymes to oxidize 1,25-dihydroxyvitamin D_3(2). Transfected cells were incubated with 1,25-dihydroxyvitamin D_3 or vehicle, and luciferase activity was measured as previously described(2). Briefly, HEK 293T cells were transiently co-transfected with Renilla luciferase to control for transfection efficiency plus the hybrid firefly luciferase reporter system, a P450-oxoreductase (P450-POR) cDNA and either empty vector, CYP24A1, or wild type or mutant CYP3A4 plasmid constructs as previously described(2). Immunoblot of CYP3A4 was performed as described as previously with minor changes(3).

To assess the ability of recombinant CYP3A4 enzymes to oxidize an alternative substrate HEK293T cells were transiently transfected as above, and were then incubated with the bioluminescent substrate luciferin isopropyl (Luciferin IPA, Promega) at a final concentration of 0.012 mM. Control wells included 20 μM of ketoconazole, a selective inhibitor of CYP3A4.

Semi-quantitative immunoblot of transfected CYP3A4 protein.
Immunoblot of CYP3A4 was performed as described as previously with minor changes(3). In brief, 25 ug of total protein from transfected cells was separated using an SDS/PAGE gradient gel (Invitrogen) and transferred onto a Nitrocellulose membrane. This was blocked for 1 h in Tris-buffered saline containing 5% nonfat dry milk, and the membrane was incubated overnight with a 1:500 dilution of polyclonal rabbit antibody to CYP3A4(4), washed and then incubated with a horseradish peroxidase conjugated secondary antibody (Catalog number # RPN4301GE Biosciences Piscataway, NJ) and developed with an enhanced chemifluorescence kit (GE Biosciences, Piscataway, NJ).
Determining reference ranges for uncommon labs
For lab reference ranges such as values for 4β,25-dihydroxyvitamin D_3/25-dihydroxyvitamin D_3, serum cholecalciferol, 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D after a 50,000 IU bolus in a rickets affected population where there are not widely used lab reference ranges, we used published values(5) and unpublished values from our recent manuscript(5) and a method described by Hozo, et al(6) for estimation of the mean and standard deviation from the median and range and the reference range was defined as the mean ± 2 standard deviations.

More detailed information regarding the clinical course of each subject.

Subject 1.1: Our first subject was remarkable for the absence of any recognizable risk factors for vitamin D deficiency rickets. At two and a half years old, this anglosaxon girl was initially referred to a paediatric endocrinologist (CR) by her local rural paediatrician who had diagnosed rickets (25 OH vit D < 16 nmol/l; TCa 1.67mmol/l (2.2 - 2.6); PO4 1.14mmol/l; ALP 2,826iu/l; PTH 58.3pmol/l) and had given her oral calcitriol 0.5mcg daily with calcium supplements for one month with no improvement in her rickets. Her parents had raised concerns about her "bow legs" and unsteady gait from aged 20 months. She had an uneventful birth with a birth weight of 7lbs 12ozs and was formula fed. On specific questioning her parents reported that she had had several episodes of brief spasms in her upper and lower limbs during sleep. She had been otherwise active and healthy, and spent much of the day outdoors. Both of

her parents and her older brother were healthy, and there was no family history of calcium or skeletal disorders. Dietary history revealed adequate dietary calcium intake and a generally well balanced diet. On examination, her height of 81.3 cm was between 3rd -10th centile and her weight was 11.5kg (25 – 50th%), she had palpable rachitic rosary, with bilateral wrist swelling, leg bowing and left tibial torsion.

A presumptive diagnosis of vitamin D resistant rickets made (due to presumed vitamin D receptor mutation), and she was commenced on oral calcitriol and doses were increased over 2 months from 0.5 – 1.5mcg/day, with no improvement in her serum calcium.

A vitamin D generation test(7) was then administered giving Calciferol 600,000 iu via nasogastric tube as an inpatient. Five days later her total serum calcium increased to near normal to 2.02 mmol/l (2.2 – 2.6) and her 25 OH vitamin D increased to 97 nmol/l, excluding 25 hydroxyalse deficiency.

Following this testing she was given weekly intravenous calcitriol 0.25 mcg increasing to calcitriol 12 mcg thrice weekly over 2 months together with calcium supplementation 1.8gm orally daily. As a result, her calcium normalised, alkaline phosphatase decreased and rachitic changes improved radiologically. However, the local hospital was unable to provide an ongoing budget for the large doses of parenteral calcitriol required.

On review of her Vitamin D generation test and noting her improved calcium on a bolus dose of 600,000 IU calciferol orally, she was commenced on a trial of calciferol syrup 50,000iu daily. At 8 years of age after five and a half years of treatment with calciferol 20,000 – 50,000iu/day, her leg bowing had resolved, with sustained resolution of her rickets biochemically and radiologically. Her height centiles had also increased to 25 – 50th % more appropriate for her mid parental height around the 25th centile. She continued on high dose calciferol for another 3 – 4 years. At aged 13 years she had not taken calciferol for over a year, she had undergone normal puberty and was menstruating regularly. She was then lost to follow-up for several years and then she re-presented complaining of bone pain. We undertook genetic and biochemical testing as described in this paper when she was aged 20 years.

Subject 2.1

On presentation at the age of 16 in England she was being treated with 4 mcg of calcitriol daily and 500 mg of elemental calcium. X-rays of her wrists (Figure S1) revealed osteopenia and metaphyseal lucency. She had a low serum calcium of 1.87 an elevated PTH (75 pmol.L (normal range 1.6-6.9) and a low 25-hydroxyvitamin D (16.5 nmol/L). Calcitriol and calcium were stopped and Ergocalciferol 15,000 units per day was initiated with no improvement in results. Oral Ergocalciferol was stopped and due to physician concern about compliance she was given IM Ergocalciferol 300,000 units with no improvement. Bone pain worsened and a trial of oral phosphate supplementation with alfacalcidol led to spontaneous insufficiency fractures of the proximal tibia and pelvis. She was then eventually transitioned to 50,000 IU of cholecalciferol daily with normalization in her labs.

Genomic location	Function	Gene	MAF in 1000 Genomes Project	MAF in ESP6500SI	dbSNP	SIFT	Polyphen2	Annotation	Occurrence in ExAC
Subject 1.1									
1:16736367	Missense	*SPATA21*	NA	0.000077	NA	0	0.996	SPATA21:NM_198546:exon6:c.316C>T:p.R106W	6
1:43102988	Missense	*CCDC30*	NA	NA	NA	0.05	0.284	CCDC30:NM_001080850:exon11:c.1577G>A:p.R526Q	2
1:78180308	Missense	*USP33*	NA	0.000077	rs139228998	0.61	0	USP33:NM_201624:exon19:c.2206A>G:p.I736V	2
10:104678284	Missense	*CNNM2*	NA	0.00008	NA	0	0.402	CNNM2:NM_017649:exon1:c.47G>A:p.G16E	3
11:2291058	Missense	*ASCL2*	NA	NA	NA	0.19	0.941	ASCL2:NM_005170:exon1:c.505G>A:p.D169N	NA
11:68370941	Missense	*PPP6R3*	NA	NA	NA	0.01	0.985	PPP6R3:NM_001164163:exon21:c.2344G>A:p.E782K	NA
12:55361669	Missense	*TESPA1*	NA	NA	NA	0.3	NA	TESPA1:NM_001098815:exon4:c.214G>A:p.E72K	NA
14:24676521	Stopgain	*TSSK4*	NA	NA	NA	NA	NA	TSSK4:NM_001184739:exon3:c.641dupA:p.Y214_A215delinsX	NA
16:2230430	Missense	*CASKIN1*	NA	NA	NA	0.24	0.996	CASKIN1:NM_020764:exon18:c.2939G>A:p.R980Q	2
16:88788037	Missense	*PIEZO1*	NA	NA	NA	0.02	NA	PIEZO1:NM_001142864:exon38:c.5312C>T:p.P1771L	4
17:7726902	Missense	*DNAH2*	NA	0.000154	NA	0.14	0.601	DNAH2:NM_020877:exon74:c.11285A>C:p.H3762P	6
18:19424179	Missense	*MIB1*	NA	NA	NA	NA	0.059	MIB1:NM_020774:exon15:c.2176A>G:p.K726E	NA
18:30825229	Missense	*CCDC178*	NA	0.000154	NA	0.05	0.868	CCDC178:NM_001105528:exon14:c.1573G>C:p.E525Q	3
19:14070195	Missense	*DCAF15*	NA	0.000078	rs141014315	0.07	0.565	DCAF15:NM_138353:exon7:c.1123C>A:p.P375T	4
19:14094305	Missense	*RFX1*	NA	0.000231	rs144348762	0	0.509	RFX1:NM_002918:exon3:c.422C>T:p.T141I	NA
2:98274553	Missense	*ACTR1B*	NA	NA	NA	0	0.997	ACTR1B:NM_005735:exon8:c.778G>A:p.E260K	NA
3:47451299	Missense	*PTPN23*	NA	0.000308	NA	0.11	0.019	PTPN23:NM_001304482:exon18:c.1726G>A:p.A576T	10
4:183674671	Missense	*TENM3*	NA	NA	NA	0	NA	TENM3:NM_001080477:exon21:c.3931A>T:p.N1311Y	NA
4:48158705	Missense	*TEC*	NA	0.000154	rs147079314	0	0.895	TEC:NM_003215:exon9:c.784C>T:p.R262C	9
4:7774696	Missense	*AFAP1*	NA	0.000077	NA	0.01	0.001	AFAP1:NM_198595:exon15:c.1852	1

								C>G:p.L618V	
4:87695632	Missense	*PTPN13*	NA	NA	NA	0.19	NA	PTPN13:NM_080684:exon30:c.4883A>C:p.Q1628P	NA
6:32011583	Missense	*TNXB*	NA	NA	NA	0	0.324	TNXB:NM_032470:exon4:c.760G>A:p.V254M	NA
6:33650398	Missense	*ITPR3*	NA	NA	NA	0	0.988	ITPR3:NM_002224:exon34:c.4574G>A:p.C1525Y	NA
7:99361602	Missense	*CYP3A4*	NA	NA	NA	0.04	0.013	CYP3A4:NM_017460:exon10:c.902T>C:p.I301T	NA
8:22054882	Missense	*BMP1*	NA	NA	NA	0.03	0.006	BMP1:NM_001199:exon15:c.2056A>C:p.K686Q	NA
8:3611554	Missense	*CSMD1*	NA	0.000084	NA	0.13	NA	CSMD1:NM_033225:exon6:c.829A>G:p.M277V	5
9:100840592	Missense	*NANS*	NA	NA	NA	0	0.999	NANS:NM_018946:exon4:c.566C>T:p.P189L	NA
9:133755997	Missense	*ABL1*	NA	0.000077	rs143152951	0.02	0.303	ABL1:NM_005157:exon10:c.1624A>G:p.R542G	4
X:119005135	Missense	*RNF113A*	NA	NA	NA	0.05	0.937	RNF113A:NM_006978:exon1:c.442G>C:p.E148Q	5
Subject 2.1									
1:155920682	Missense	*ARHGEF2*	NA	NA	NA	0	0.98	ARHGEF2:NM_001162383:exon20:c.2641G>C:p.D881H	NA
1:248039607	Missense	*TRIM58*	NA	NA	NA	0.01	0.651	TRIM58:NM_015431:exon6:c.1277A>G:p.N426S	5
1:28099852	Nonframeshiftdeletion	*STX12*	NA	NA	NA	NA	NA	STX12:NM_177424:exon1:c.35_43del:p.12_15del	NA
10:17127758	Missense	*CUBN*	NA	NA	NA	0.03	0.817	CUBN:NM_001081:exon16:c.1948A>C:p.I650L	NA
12:57861129	Missense	*GLI1*	NA	NA	NA	0.09	0.075	GLI1:NM_001160045:exon7:c.542G>A:p.R181Q	4
14:32902937	Missense	*AKAP6*	NA	NA	NA	0	0.98	AKAP6:NM_004274:exon2:c.238A>T:p.T80S	8
14:36988255	Missense	*NKX2-1*	NA	NA	NA	0.09	0.923	NKX2-1:NM_003317:exon1:c.308C>T:p.T103I	NA
14:45673638	Missense	*MIS18BP1*	NA	NA	NA	0.02	0.002	MIS18BP1:NM_018353:exon16:c.3259A>G:p.T1087A	2
14:77493442	Missense	*IRF2BPL*	NA	NA	NA	0.06	0.649804	IRF2BPL:NM_024496:exon1:c.694	1

								C>T:p.H232Y	
16:1245507	Missense	*CACNA1H*	NA	NA	NA	1	NA	CACNA1H:NM_001005407:exon4:c.487C>A:p.Q163K	NA
16:20975614	Missense	*DNAH3*	NA	NA	NA	0.17	0.999	DNAH3:NM_017539:exon53:c.9592C>T:p.P3198S	2
16:841161	Missense	*CHTF18*	NA	NA	NA	0.23	NA	CHTF18:NM_022092:exon8:c.895T>C:p.F299L	1
19:11565429	Missense	*ELAVL3*	NA	NA	NA	0.42	0.005	ELAVL3:NM_001420:exon7:c.1016C>T:p.A339V	NA
19:33464993	Missense	*FAAP24*	NA	NA	NA	0.07	0.868	FAAP24:NM_152266:exon4:c.271G>A:p.V91I	2
19:41939286	Missense	*ATP5SL*	NA	NA	NA	0	0.999	ATP5SL:NM_001167871:exon4:c.406G>A:p.D136N	1
19:49138861	Missense	*DBP*	NA	NA	NA	0	0.654	DBP:NM_001352:exon2:c.526G>T:p.G176W	NA
19:49142861	Missense	*CA11*	NA	NA	NA	0	0.998	CA11:NM_001217:exon6:c.585C>A:p.N195K	NA
2:20202940	Missense	*MATN3*	NA	NA	NA	0.07	NA	MATN3:NM_002381:exon3:c.898G>A:p.D300N	8
21:45089781	Missense	*RRP1B*	NA	NA	NA	0.32	0.993	RRP1B:NM_015056:exon2:c.147A>C:p.E49D	2
22:25282573	Missense	*SGSM1*	NA	NA	NA	0.82	NA	SGSM1:NM_001098497:exon16:c.1648G>A:p.E550K	NA
3:111764691	Missense	*TMPRSS7*	NA	NA	NA	0.12	NA	TMPRSS7:NM_001042575:exon4:c.253G>A:p.A85T	NA
3:156396201	Missense	*TIPARP*	NA	NA	NA	0.01	0.963	TIPARP:NM_001184717:exon2:c.715G>A:p.G239R	NA
3:165491281	Missense	*BCHE*	NA	NA	NA	0.22	0.576	BCHE:NM_000055:exon4:c.1698A>T:p.E566D	NA
3:47164922	Missense	*SETD2*	NA	NA	NA	0	NA	SETD2:NM_014159:exon3:c.1204C>T:p.R402W	2
3:49039018	Missense	*P4HTM*	NA	0.000077	rs146471142	0.64	0	P4HTM:NM_177938:exon3:c.584G>A:p.R195Q	3
5:37309320	Missense	*NUP155*	NA	NA	NA	0.32	0	NUP155:NM_001278312:exon23:c.2486A>G:p.K829R	NA
7:64168571	Missense	*ZNF107*	NA	NA	NA	0	0.999	ZNF107:NM_001282360:exon3:c.2000A>G:p.H667R	NA
7:73008681	Missense	*MLXIPL*	NA	NA	NA	0	0.983	MLXIPL:NM_032951:exon16:c.2363C>T:p.T788M	1

7:99361602	Missense	*CYP3A4*	NA	NA	NA	0.04	0.013	CYP3A4:NM_017460:exon10:c.902T>C:p.I301T	NA
8:144810594	Missense	*FAM83H*	NA	NA	NA	0	0.979	FAM83H:NM_198488:exon5:c.1037C>A:p.P346Q	4
8:144999415	Missense	*PLEC*	NA	0.000096	NA	0.09	0.58463	PLEC:NM_201378:exon31:c.4640G>A:p.R1547Q	8
9:139996087	Missense	*MAN1B1*	NA	NA	NA	0.01	0.998	MAN1B1:NM_016219:exon8:c.1217G>A:p.R406Q	2
9:35958045	Missense	*OR2S2*	NA	NA	NA	0.01	0	OR2S2:NM_019897:exon1:c.51G>T:p.R17S	NA
9:5823956	Missense	*ERMP1*	NA	NA	NA	0	1	ERMP1:NM_024896:exon4:c.814C>T:p.R272C	NA
9:71836309	Nonframeshiftinsertion	*TJP2*	NA	NA	NA	NA	NA	TJP2:NM_001170415:exon5:c.861_862insAGCCGC:p.R287delinsRSR	9
X:129837181	Missense	*ENOX2*	NA	NA	NA	0.49	0.998	ENOX2:NM_001281736:exon3:c.10C>T:p.P4S	NA

References

1. Pratt-Hyatt, M., Zhang, H., Snider, N.T., and Hollenberg, P.F. 2010. Effects of a commonly occurring genetic polymorphism of human CYP3A4 (I118V) on the metabolism of anandamide. *Drug Metab Dispos* 38:2075-2082.
2. Thacher, T.D., Fischer, P.R., Singh, R.J., Roizen, J., and Levine, M.A. 2015. CYP2R1 Mutations Impair Generation of 25-hydroxyvitamin D and Cause an Atypical Form of Vitamin D Deficiency. *J Clin Endocrinol Metab*:jc20151746.
3. Quinney, S.K., Malireddy, S.R., Vuppalanchi, R., Hamman, M.A., Chalasani, N., Gorski, J.C., and Hall, S.D. 2013. Rate of onset of inhibition of gut-wall and hepatic CYP3A by clarithromycin. *Eur J Clin Pharmacol* 69:439-448.
4. Wang, R.W., and Lu, A.Y. 1997. Inhibitory anti-peptide antibody against human CYP3A4. *Drug Metab Dispos* 25:762-767.
5. Thacher, T.D., Fischer, P.R., Obadofin, M.O., Levine, M.A., Singh, R.J., and Pettifor, J.M. 2010. Comparison of metabolism of vitamins D2 and D3 in children with nutritional rickets. *J Bone Miner Res* 25:1988-1995.
6. Hozo, S.P., Djulbegovic, B., and Hozo, I. 2005. Estimating the mean and variance from the median, range, and the size of a sample. *BMC Med Res Methodol* 5:13.
7. Cipriani, C., Romagnoli, E., Scillitani, A., Chiodini, I., Clerico, R., Carnevale, V., Mascia, M.L., Battista, C., Viti, R., Pileri, M., et al. 2010. Effect of a single oral dose of 600,000 IU of cholecalciferol on serum calciotropic hormones in young subjects with vitamin D deficiency: a prospective intervention study. *J Clin Endocrinol Metab* 95:4771-4777.