Du-Supplemental Fig. 1

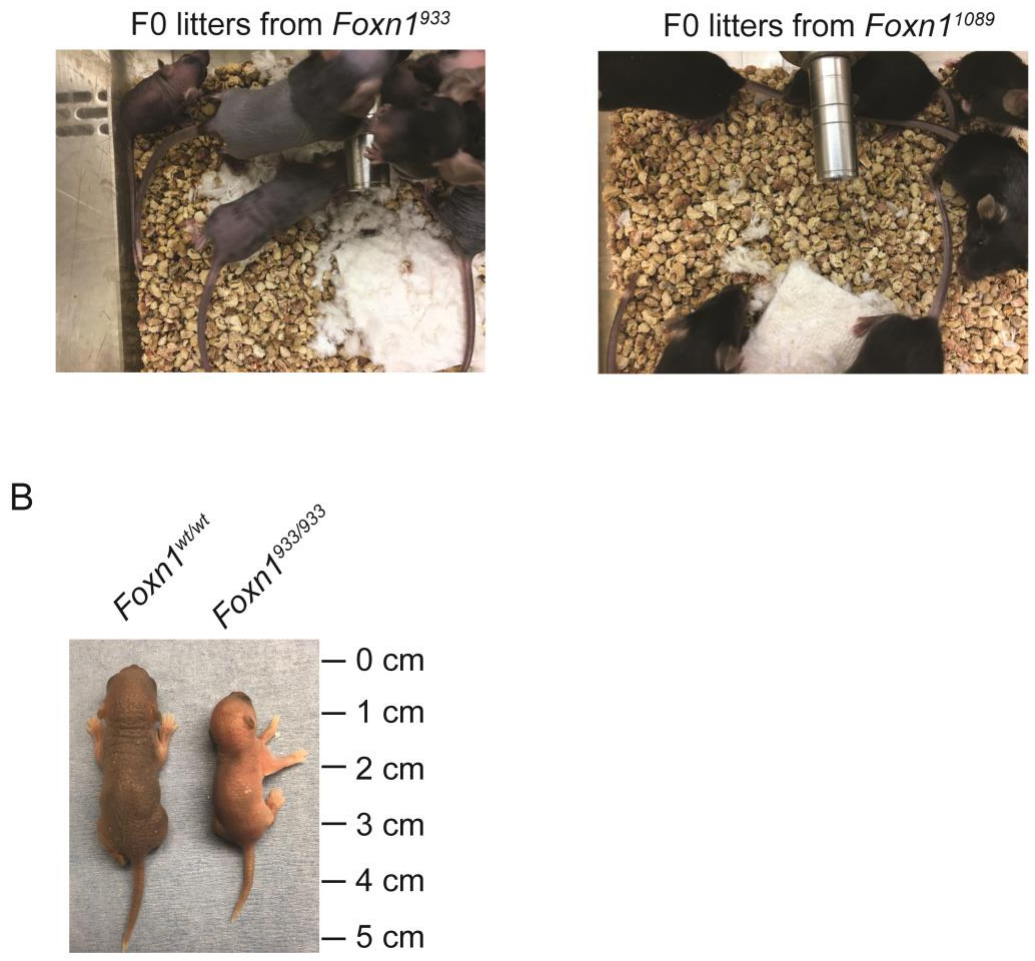

Figure S1. Founder litters (F0) from diverse *Foxn1* mutant mice only exhibit a *nu/nu* phenotype with indels or point mutations impacting both alleles affecting the DNA binding domain. A. The human *FOXN1* mutations for Pt.1 were introduced into the murine genome by CRISPR/Cas9 technologies. The DNA repair template used for each allele is shown in Figure 2A. F0 littermates from *Foxn1*^{933} mice had a range of phenotypes including those that were nude, lacked whiskers and nails, and had a small size. DNA from the nude pups was sequenced, which revealed diverse indels and/or deletions within the DNA bindings domain that impacted both alleles. B. Wild-type Foxn1 (*Foxn1*$^{wt/wt}$) and those with a nude phenotype were necropsied, revealing a thymic aplasia selectively in those with a nude appearance. C. The mice lacking a functional Foxn1 have a small, runted appearance compared to littermate controls.

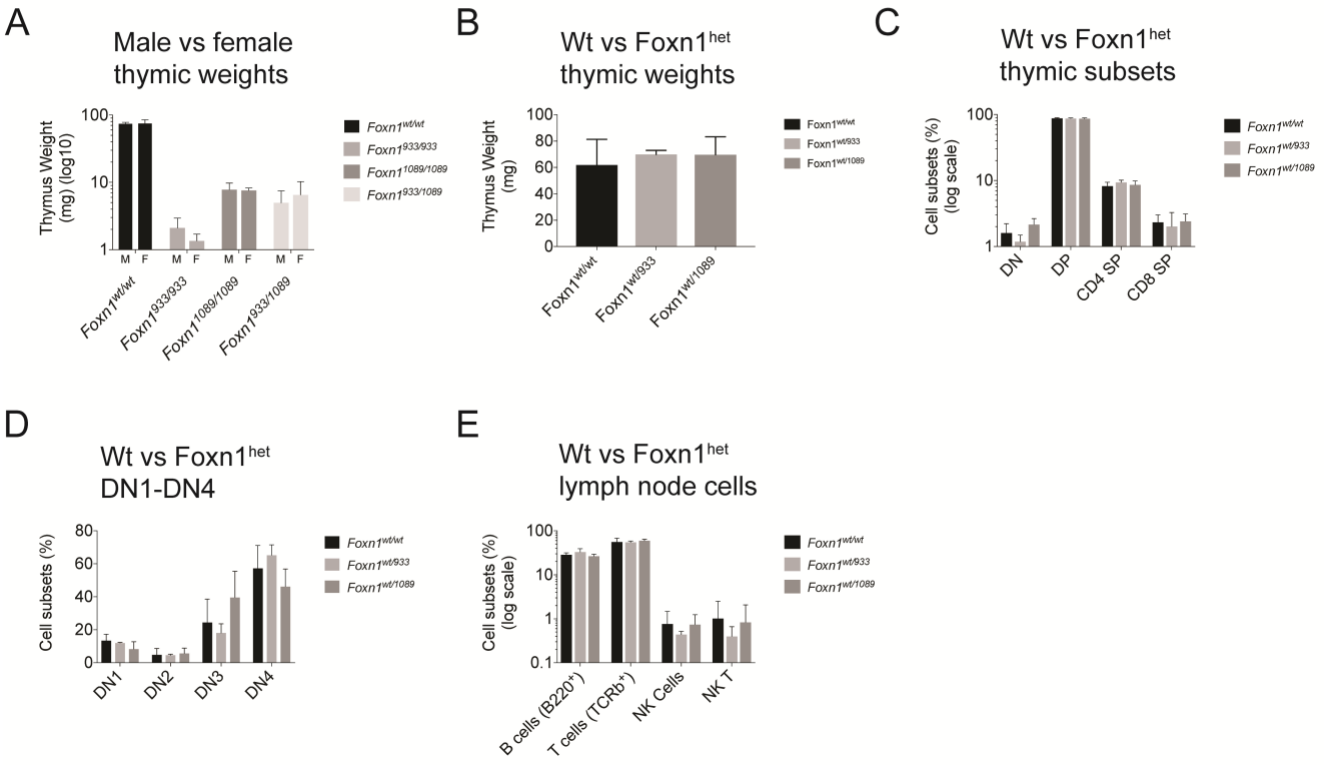

Figure S2. A thymic hypoplasia results from compound heterozygous mutations in *Foxn1* that is independent of sex and is not revealed in heterozygous mice. A. The thymus weight was calculated from the various control, heterozygous, and compound heterozygous mice was compared between male and female mice. The mice included wild-type Foxn1 (*Foxn1*$^{wt/wt}$), homozygous mutant (*Foxn1*$^{933/933}$, *Foxn1*$^{1089/1089}$), and compound heterozygous mice (*Foxn1*$^{933/1089}$), the latter matching Pt.1. The lines were obtained by intercrossing the *Foxn1*$^{wt/933}$ and *Foxn1*$^{wt/1089}$ lines. Data are representative of mean +/- SEM from between 8 and 15 mice per group. There were no statistically significant differences, determined with students t-test and two-way ANOVA. B. Wild-type Foxn1 (*Foxn1*$^{wt/wt}$), and heterozygous mutant mice (*Foxn*$^{wt/933}$, *Foxn1*$^{wt/1089}$) were obtained by intercrossing the *Foxn1*$^{933/wt}$ and *Foxn1*$^{1089/wt}$ lines. The thymus and lymph nodes were isolated and single cell suspensions prepared for flow cytometry. B. The thymus weight was calculated for the various control and heterozygous mice. C. Fluorochrome labeled antibodies against CD4, CD8 were used to detect DN, DP, and SP thymocyte subsets. D. The progression of thymocytes from the DN1-DN4 stages of thymocytes was assessed by cell surface staining for CD44 and CD25 following exclusion of cells expressing CD3, CD4, CD8, B220, NK1.1, TCR$\gamma\delta$, Ter199, CD11b, and CD11c. E. Lymph nodes were collected from the indicated mice and stained with antibodies detecting cell surface CD4, CD8, B220, TCR beta, and NK1.1. The cells were analyzed by flow cytometry comparing the percentage of cells epxressing B220 (B cells), TCRb (T cells), and NK cells. For experiments in A-E, bar graphs reveal the percentage of various subsets, determined from pooled experiments using a minimum of 5 mice/line. Data are representative of mean +/- SEM with 5-15 mice per group. There were no statistically significant differences noted in the comparisons among the wild-type and heterozygous mice.

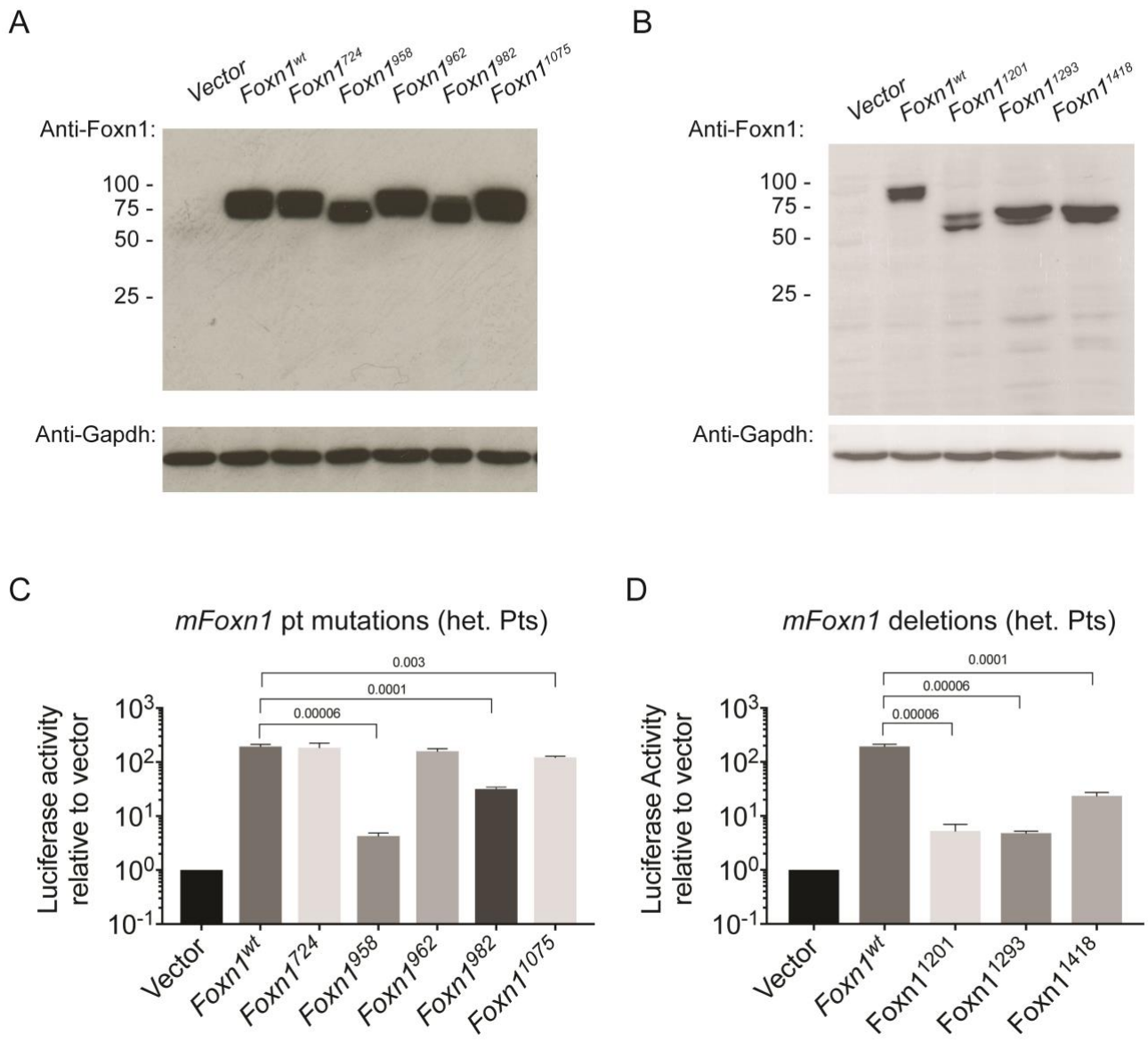

Figure S3. Diverse mutations in *FOXN1* lead to differential effects on protein expression and function. A-B. Transient transfection assays in HEK293 T cells were used with expression vectors for wild-type *Foxn1* or *Foxn1* constructs harboring the indicated mutations that matched those *FOXN1* identified in human patients. Single allelic mutations were introduced into the murine Foxn1 cDNA. pCMV-FLAG plasmids were transfected into HEK293T cells. Forty-eight hrs. post transfection, the cells were lysed in RIPA buffers, proteins extracted and resolved by SDS-PAGE. Western blotting was performed with antibodies specific for the N-terminal region of FOXN1, followed by antibodies detecting Gapdh, which was used as a loading control. Blots are representative of 2-5 independent experiments. C-D. HEK293T cells were transfected with the indicated constructs along with a *Psmb11* luciferase reporter construct in combination with a beta-galactosidase plasmid for normalization purposes. Forty-eight hrs. post-transfection, the cells were harvested and luciferase and b-galactosidase activity was measured. The luciferase activity was normalized to beta-galactose used as an internal control. Data are representative of 3-5 independent experiments. P values were determined. * = 0.05, *** = 0.001, **** = 0.0001.

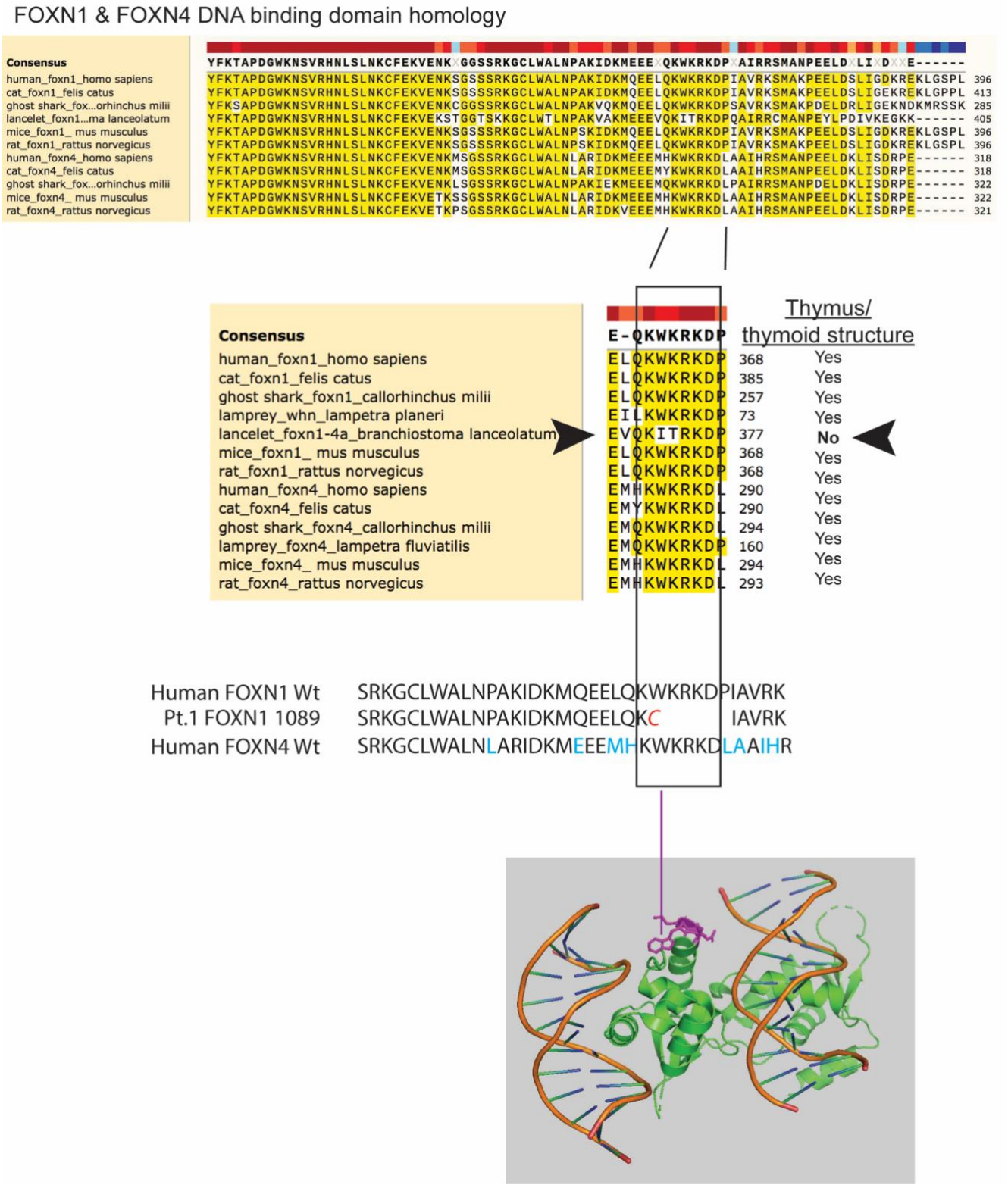

Figure S4. The Foxn1 15-nucleotide region near 1089 reveals substantial sequence homology to species with evidence of a thymus/thymoid-like structure. The amino acid sequence homology of Foxn1 and Foxn4 from the species indicated was compared for the segment comprising the DNA binding domain. A 5-amino acid sequence, deleted in FOXN1 from Pt. 1, was compared with different FOXN1/4 family members. This sequence is highly conserved in species with evidence of a thymus or thymoid-like structure. The sequence differs in lancelets, a

cephalochordate that lacks a thymoid (closed arrows). The crystal structure and the location of the W within the 5-amino acid sequence is indicated with an arrow. A full-length protein crystal has not yet been published.

Table S1. Clinical and immunological presentations of patients identified with single *FOXN1* mutations

	Age	ALC (cells/μL)	CD3 (cells/μL)	CD4 (cells/μL)	CD8 (cells/μL)	CD4, CD45RA$^+$ (% of CD4)	CD4, CD45RA$^+$ (cells/μL)	NK (cells/μL)	CD19 (cells/μL)	Mitogen proliferation
Reference Ranges Ref (1)	0-3 m	3400 - 7600	2500 – 5500	1600 – 4000	560 – 1700	64-95	1200 – 3700	170 – 1100	300 – 2000	Normal PHA Response >75,000 cpm
	3-6 m	3900 - 9000	2500 - 5600	1800 - 4000	590 - 1600	77-94	1300 - 3700	170 - 830	430-3000	
	6-12 m	3400 – 9000	2500 – 5600	1900 – 5900	1040 – 1700	64-93	1100 – 3700	160 – 950	610 – 2600	
	1-2 y	3600 – 8900	2100 – 6200	1300 – 3400	620 – 2000	63 - 91	1000 – 2900	180 – 920	720 – 2600	
Pt.3	52 days	-	265	228	40	41.7	111	608	19	-
	69 days	-	-	235	-	-	-	-		-
	134 days	-	450	381	78	29.5	133	608	1970	-
Pt.4	10 days	2600	468	360	52	-	-	520	702	-
	34 days	1800	228	190	38	-	-	342	1197	abnormal
	51 days	2500	265	228	40	41.7	111	608	1600	normal
	133 days	3100	450	381	78	29.5	133	608	1970	-
	185 days	2900	557	479	75	21.6	120	464	1810	-
	276 days	4700	2359	682	1734	4.2	99	1081	1230	-
	395 days	-	563	370	172	15.2	86	489	990	-
Pt.5	59 days	-	1239	1071	168	46	744	168	567	
	80 days	-	1212	977	217	-	-	300	300	-
	146 days	-	-	-	-	-	-	-	-	normal
Pt.6	8 days	4400	1572	1007	617	-	-	847	1005	abnormal
	14 days	-	1242	714	511	-	-	647	776	-
	84 days	4690	1670	1037	587	-	-	981	1825	abnormal

	336 days	4890	2254	1352	813	-	-	1303	1166	normal
Pt.7	~ 5 m	-	62	22	23	-	29	-	-	-
	~ 7 m	2400	32	13	9	4.6	1	105	1280	absent
Pt.8	6 days	2033	980	700	280	-	-	240	660	normal
Pt.9	41 days	1716	274	156	106	9	154	502	918	normal
	111 days	1599	225	144	65	-	-	558	880	-
	209 days	1472	357	231	112	9	132	364	926	normal
	~ 12 m	6200	296	180	90	19.4	1202	896	812	normal
	~ 18 m	6900	544	340	156	15.4	1062	814	1476	normal
	~ 24 m	7200	444	293	127	16.4	1180	777	740	-
	~ 30 m	4800	430	281	112	11.9	571	151	483	normal
Pt.10	12 days	2400	650	516	142	50	325	1058	630	normal
	26 days	2300	734	557	168	48	352	414	1150	-
	53 days	2300	860	665	182	48.6	418	228	1120	-
	88 days	2600	871	697	172	55.6	484	216	1470	-
	116 days	2000	686	518	146	48.9	335	164	1130	-
	205 days	2500	813	598	185	47.4	385	230	1380	-
	282 days	2900	1218	835	322	43.3	527	207	1370	-
	387 days	2700	1164	815	289	44.3	516	251	1260	normal
	477 days	2700	1175	745	605	32.5	382	238	1270	normal
Pt.11	14 days	2420	73	70	0	-	-	-	-	-
	22 days	-	-	-	-	-	-	-	-	normal
	54 days	1200	156	136	12	-	-	-	888	normal
	12 months	2346	657	493	117	-	-	336	1361	normal
	18 months	2063	598	371	144	-	-	268	887	normal

	24 months	2464	789	552	148	-	-	296	1158	normal
	31 months	2477	991	666	198	-	-	648	1065	normal
Pt.12	27 days	2210	930	782	135	23.7	524	398	824	normal
	53 days	2560	1167	970	161	28.4	727	351	968	
	73 days	3120	1289	1067	175	23.2	724	309	1432	
	104 days	3330	1395	1099	233	23.6	786	543	1335	
	227 days	4420	1830	1339	415	23.7	1048	424	2148	
	377 days	4145	1699	1256	344	19.9	825	642	1753	
	496 days	4470	1851	1269	447	17.1	764	407	2172	
Pt.13	9 days	3500	538	464	81	-	-	1970	707	-
	31 days	3920	706	545	125	-	-	1917	1051	-
	12 months	-	943	655	204	-	-	1413	1917	normal
Pt.14	61 days	-	463	337	91	-	-	607	2058	-
	89 days	-	359	249	85	-	-	234	1792	-
	118 days	-	409	300	81	-	-	128	1471	-
	181 days	-	463	337	91	-	-	191	1441	-
Pt.15	29 days		63	58	5	-	-	282	1207	abnormal
	36 days		53	48	2	-	-	440	1375	-
Pt.16	5 days	-	7	1	1	1	5	223	382	-
	9 days	-	11	3	4	1	12	364	508	-
	46 days	-	-	-	-	-	-	-	-	normal
	64 days	-	107	93	14	1	8	248	668	-
	87 days	-	91	81	9	2	15	179	706	-

1. Shearer WT, Rosenblatt HM, Gelman RS, Oyomopito R, Plaeger S, Stiehm ER, et al. Lymphocyte subsets in healthy children from birth through 18 years of age: the Pediatric AIDS Clinical Trials Group P1009 study. *The Journal of allergy and clinical immunology.* 2003;112(5):973-80.

Table S2. Heterozygous and compound heterozygous mutations identified in human *FOXN1*					
Identifier	DNA Mutation	Protein Coding Mutation	Domain Affected	Predicted Protein Length (amino acids)	Protein Function (% of WT) (Luciferase Assay)
Normal	Wild-type	N/A	N/A	1-648	100%
Pt.1	c.933_936dupACCC	p.T313fsX169	DNA binding	1-480	1.5%
Pt.1	c.1089_1103del15	p.W363C	DNA binding	1-643	31%
Pt.2	c.1288C>T	p.P430S	Transactivation	1-648	100%
Pt.2	c.1465delC	p.R489fsX61	Transactivation	1-548	18%
Pts.3-4	c.1465delC	p.R489fsX61	Transactivation	1-548	18%
Pt.5	c.724C>T	p.P242S	Exon 5	1-648	95%
Pt.6	c.958C>T	p.R320W	DNA binding	1-648	2%
Pt.7	c.962A>G	p.H321R	DNA binding	1-648	82%
Pt.8	c.982T>C	p.C328R	DNA binding	1-648	16%
Pt.9	c.1075G>A	p.E359K	DNA binding	1-648	63%
Pts.10-14	c.1201-1216	p.A406fsX144	Exons 8-9	1-453	3%
Pt.15	c.1293delC	p.P432fsX118	Exons 8-9	1-648	2%
Pt.16	c.1418delC	p.H473fsX77	Exons 8-9	1-648	12%

Table S3. Differentially expressed genes from normal and hypoplastic fetal thymic lobes				
Fetal thymic lobes from indicated genotype				
	Foxn1$^{wt/wt}$		*Foxn1*$^{933/1089}$	
Transcripts	Mean[a]	SD[a]	Mean	SD
Foxn1	373	175	134	62
Plcd1	226	35	83[b]	63
TGFB1	761	134	148	34
Fgf12	160	33	96	19
Bmpr1A	44	5	57[c]	18
Msx1	4	2	5	2
Lef1	48	28	14	2
Dsc2	1033	175	88	15
Akt1	251	55	228	121
Akt3	2590	176	5801	2665
Gata3	43	13	145	54
Pip2(Pik3CA)	173	37	150	4
Pkca (Prkca)	135	19	163	3
Pkcd (Prkcd)	445	43	404	29
Tbx1	5	2	15	9
Pax1	660	95	173	74
Pax9	314	42	227	6
HoxA3	4	1	2	1
Eya1	1766	298	10940	10449
Six1	1401	507	1225	300
Ccl25	27	6	19	1
Dll4	1186	322	86	61

[a]Changes in several key transcripts is tabulated with the average and the standard deviation of triplicate isolates/group provided.

[b,c]Green and red reflect reduced or elevated transcript expression in the hypoplastic thymii, respectively.

Supplemental Table S4. Key Reagents and Resources		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Digoxigenin-AP	Roche	11093274910
Anti-CD8-FITC	Tonbo Biotech	35-0081-U500
Anti-CD4-PE	Tonbo Biotech	50-0041-U100
Anti-TCR-b-PerCP-Cy5.5	Tonbo Biotech	65-5961-U100
Anti-CD69-APC	ebioscience	17-0691-82
Anti-CD45-PerCPCy5.5	biolegend	103132
Anti-B220-APC	Tonbo Biotech	20-0452-U100
Anti-CD44-APC	BD bioscience	559250
Anti-CD25-FITC	BD bioscience	553072
Anti-CD8-PE	Tonbo Biotech	50-0081-U100
Anti-B220-PE	BD bioscience	553090
Anti-NK1.1-PE	BD bioscience	553165
Anti-γδTCR-PE	BD bioscience	553178
Anti-CD11b-PE	BD bioscience	557397
Anti-CD11c-PE	Tonbo Biotech	50-0114-U100
Anti-CD19-PE	Tonbo Biotech	50-0193-U100
Anti-Ter-119-PE	Tonbo Biotech	50-5921-U100
Anti-FLAG mAb	Sigma Chemical	F1804
Anti-Foxn1	Santa Cruz Biotech	sc271256
Anti-gapdh	Santa Cruz Biotech	Sc47724
Bacterial and Virus Strains		
XL1-Blue Supercompetent Cells	Agilent	200236
BL21-Codon PLus	Agilent	230280
DH5 alpha	Invitrogen (Thermo-Fisher)	18265017
Biological Samples		
Embryos	C57BL/6 mice	
Thymus	C57BL/6 mice	
Skin	C57BL/6 mice	
Tails	C57BL/6 mice	
Chemicals, Peptides, and Recombinant Proteins		
Turbo DNAase	Invitrogen-Fisher	AM2238
Critical Commercial Assays		
Luciferase Assay System	Promega	E1500
MEGAscript™ T7 Transcription Kit	Invitrogen	AM1334
High Capacity cDNA synthesis kit	ABI-Thermo Fisher	4368814
FluoReporter™ lacZ/Galactosidase Quantitation Kit	Thermo Fisher	F2905
Deposited Data		
GEO Affymetrix Identifier	Mouse Clariom S	GES134458
Experimental Models: Cell Lines		
HEK 293T	ATCC	CRL-3216

OP9-Dll1 (stromal feeder cell line)	Juan Carlos Zuniga-Pflucker	
TEC427.1(thymic epithelial cell line)	Stanislas Vukmanovic	
Experimental Models: Organisms/Strains		
C57BL/6	The Jackson Laboratory	#000664
Foxn1$^{933KI/KI}$ using C57Bl/6	UTSW Transgenic Core Facility	#17 and #30 lines
Foxn1$^{1089KI/KI}$ using C57Bl/6	UTSW Transgenic Core Facility	#B4 and #B15 lines
Foxn1-Cre on C57Bl/6 background	The Jackson Laboratory	#018448
Oligonucleotides		
RT-PCR Primers, cloning primers	IDT DNA Tech	seeTable S5
Recombinant DNA plasmids		
pCMV-FLAG	Sigma Chemical	V79020
pCMV-mFoxn1-wt	van Oers Lab	#858
pCMV-mFoxn1-933	van Oers Lab	#859
pCMV-mFoxn1-1089	van Oers Lab	#860
pCMV-mFoxn1-1288	Addgene	#861
pCMV-mFoxn1-1465	van Oers lab	#862
pCMV-mFoxn1-724	van Oers lab	#863
pCMV-mFoxn1-958	van Oers lab	#864
pCMV-mFoxn1-962	van Oers lab	#865
pCMV-mFoxn1-982	van Oers lab	#866
pCMV-mFoxn1-1075	van Oers lab	#867
pCMV-mFoxn1-1201	van Oers lab	#868
pCMV-mFoxn1-1293	van Oers lab	#869
pCMV-mFoxn1-1418	van Oers lab	#870
pCMV-mFoxn1-N4 to N1	van Oers lab	#871
Software and Algorithms		
SnapGene	GSL Biotech LLC	
Graphpad Prism	Prism 7 for MAC	
Flowjo	FlowJo LLC	
PyMOl	Python License	

Supplemental Table S5. Oligonucleotide primers used for PCR, RT-PCR and qRT-PCR

Primer Name	Oligonucleotide Identifier	Nucleotide Sequence
Mouse Typing 933 F	1516	5'-TAGACAGCTCCTTCCCGATGGCTC-3'
Mouse Typing 933 R	1476	5'-AGAAAGCTTGACGCCGACAGGAAGTCC-3'
Mouse Typing 933 F (WT)	1563	5'-TAGACTGCTCCTGATGGCTGG-3'
Mouse Typing 933 R (WT)	1476	5'-AGAAAGCTTGACGCCGACAGGAAGTCC-3'
Mouse Typing 1089 F	1475	5'-TCTGGTACCACATAGTAGGCGTCAAACCC-3'
Mouse Typing 1089 R	1517	5'-GATGCTTTTCCGGACAGCTATGCA-3'
Mouse Typing 1089 F (WT)	1475	5'-TCTGGTACCACATAGTAGGCGTCAAACCC-3'
Mouse Typing 1089 R (WT)	1564	5'-CAATGGGGTCTTTCCTCTTCCA-3'
mFoxn1 933 mutagenesis	1512	5'-GCACTTCCCTTACTTCAAGACTGCTCCTTCCCGATGGCTGGCAGAACTCTGTTCGC-3'
mFoxn1 1089 mutagenesis	1473	5'-CAGGAAGAACTGCAGAAGTGCATTGCTGTGCGCAAAAGCA-3'
mFoxn1 1288 mutagenesis	1607	5'-GCACCCAATGCATCCAGCTTCAGGCCCCATGCCTG-3'
mFoxn1 1465 mutagenesis	1595	5'-CAGCCAGGCACCCCCAGGACTCACCTCTACCTG-3'
mFoxn1 724 mutagenesis	1649	5'-GGTGGAGGCAGCTACTCTGTGCCCTACCTG-3'
mFoxn1 958 mutagenesis	1651	5'-GCTGGAAGAATTCTGTTTGGCATAACCTGTCCCTCAAC-3'
mFoxn1 962 mutagenesis	1653	5'-GAAGAATTCTGTTCGCCGTAACCTGTCCCTCAAC-3'
mFoxn1 982 mutagenesis	1655	5'-CTGTCCCTCAACAAGCGCTTTGAGAAGGTGG-3'
mFoxn1 1075 mutagenesis	1657	5'-GACAAGATGCAGGAAAAACTGCAGAAGTGG-3'
mFoxn1 1201 mutagenesis	1691	5'-GGCTCTCCGCTGCTGGGCTGTCCAGCCCAGGTCCCATCCGGCCCATGGCACC-3'
mFoxn1 1293 mutagenesis	1693	5'-CAATGCATCCAGCTCCAGGCCCATGCCTGGCAAG-3'
mFoxn1 1418 mutagenesis	1695	5'-CCAGCAGCCATTGTTCCACAGCCAGATGGGCATC-3'

mFoxn1 Seq Primer-864	1699	5’-GACCGGAAGCCTTCCAGTCAG-3’
Human FOXN1 Exon 8 F	1736	5’-GAGCTGGACAGCCTCATTGGAG-3’
Human FOXN1 Exon 8 R	1737	5’-CCTGGAAGTCGAAGTCAGTGAG-3’
mGapdh F	1583	5’-AGGTCGGTGTGAACGGATTTG-3’
mGapdh R	1584	5’-TGTAGACCATGTAGTTGAGGTCA-3’
mKrt84 F	1665	5’-TCAGCATCTGAACCGCTTCC-3’
mKrt84 R	1666	5’-TTCTGTAGCCAAAGCCAGG-3’
mKrt32 F	1667	5’-CAGCTGCCTTTCTAAGACCTAC-3’
mKrt32 R	1668	5’-TCATTCAGGACCTGCATGGTC-3’
mDsg4 F	1669	5’-CACCTTACGGAGTGTTCAC-3’
mDsg4 R	1670	5’-GGTTGTCGTTCACATCCATGAC-3’
mKrt86 F	1679	5’-GGAGCAGAGGTTGTGTGAGG-3’
mKrt86 R	1680	5’-AGGGGCAGTACCAGAGACG-3’
mHox13 F	1609	5’-ATGACGACTTCGCTGCTCCT-3’
mHox13 R	1610	5’-GGCGGTGAGATTAGGGGTGG-3’
mFoxq1 F	1615	5’-CGAGATCAACGAGTACCTCATGG-3’
mFoxq1 R	1616	5’-GCATCCAGTAGTTGTCCTTGCC-3’
mKrt33b F	1677	5’-CCTGGACGAACTGACCCTCT-3’
mKrt33b R	1678	5’-CATGGTTTTGCTTGAGACACAG-3’
mDsc2 F	1663	5’-ATTCATTGGGTCCCTTCCCAC-3’
mDsc2 R	1664	5’-CCAGTGCAATACAGGTTTCCAG-3’