Research Article

Sequence Variation and Expression of the *Gimap* Gene Family in the BB Rat

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Positional cloning of lymphopenia (*lyp*) in the BB rat revealed a frameshift mutation in *Gimap5*, a member of at least seven related GTPase Immune Associated Protein genes located on rat chromosome 4q24. Our aim was to clone and sequence the cDNA of the BB diabetes prone (DP) and diabetes resistant (DR) alleles of all seven *Gimap* genes in the congenic DR.*lyp* rat line with 2 Mb of BB DP DNA introgressed onto the DR genetic background. All (100%) DR.$^{lyp/lyp}$ rats are lymphopenic and develop type 1 diabetes (T1D) by 84 days of age while DR.$^{+/+}$ rats remain T1D and *lyp* resistant. Among the seven *Gimap* genes, the *Gimap5* frameshift mutation, a mutant allele that produces no protein, had the greatest impact on lymphopenia in the DR.$^{lyp/lyp}$ rat. *Gimap4* and *Gimap1* each had one amino acid substitution of unlikely significance for lymphopenia. Quantitative RT-PCR analysis showed a reduction in expression of all seven *Gimap* genes in DR.$^{lyp/lyp}$ spleen and mesenteric lymph nodes when compared to DR.$^{+/+}$. Only four; *Gimap1*, *Gimap4*, *Gimap5*, and *Gimap9* were reduced in thymus. Our data substantiates the *Gimap5* frameshift mutation as the primary defect with only limited contributions to lymphopenia from the remaining *Gimap* genes.

1. Introduction

Lymphopenia (*lyp*) is a prerequisite for spontaneous type 1 diabetes (T1D) in the BioBreeding (BB) diabetes prone (DP) rat [1]. Positional cloning of the *lyp* gene revealed a frame shift mutation in *Gimap5* (previously known as *Ian5* or *Ian4L1*). *Gimap5* is a member of at least seven related GTPase Immune Associated Protein (*Gimap*) genes located within 150 Kilobases (Kb) on rat chromosome (RNO) 4 [2, 3]. DR.$^{lyp/lyp}$ rats, where 2 Mb of DP DNA was introgressed onto the BB diabetes resistant (DR) genetic background, are lymphopenic and 100% develop spontaneous T1D by 84 days of age [4].

The positional cloning and subsequent identification of the *Gimap5* gene on RNO4 were in part established through generation of the DR.*lyp* congenic rat line along with recombination events following our method of marker assisted breeding of DP with F344 rats [2, 4, 5]. Analysis of the *lyp* phenotype in the F344 DNA recombinant rats helped us define the critical *lyp* interval as a region of approximately 33 Kb between D4Rhw6 (76.83 Mb) and IIsnp3 (77.16 Mb) containing *Gimap1*, *Gimap5*, and *Gimap3* (formerly known as *Ian2*, *Ian5*, and *Ian4*, resp.) [2, 4]. *Gimap5* was identified as the *lyp* gene in the BBDP rat through a frameshift mutation and premature truncation of the Gimap5 protein [2, 6]

and can be rescued in a P1-derived artificial chromosome (PAC) transgenic rat [7]. However, potential contributions to lymphopenia and/or T1D from the other *Gimap* genes are still unknown. Similarly, how the mechanisms by which reduced *Gimap5* transcript levels and the absence of the Gimap5 protein [2, 7, 8] contribute to lymphopenia and T1D are still being elucidated [9–13].

The predicted structures of the Gimap proteins show common sequences and motifs, such as GTP-binding domains in the N-terminal half, but with differing C-terminal ends [2, 3]. Some C-terminal regions are consistent with transmembrane domains as in the case of Gimap1 and Gimap5, while others, as in Gimap9 and Gimap4, predict coiled coil domains [3, 14]. Both GIMAP4 and GIMAP7 from human Jurkat cells [3] localize to the endoplasmic reticulum and Golgi apparatus while mouse Gimap3 from murine IL-3-dependent 32D myeloid precursor cells was expressed at the outer mitochondrial membrane [15]. Conflicting reports show that GIMAP5, from human primary T cells [10] and from GIMAP5 transfected 293T cells [16], localizes to the centrosome, Golgi apparatus, or endoplasmic reticulum (ER), whereas Gimap5, cloned from Rat2 fibroblasts, localizes to a distinct subcellular fraction that is neither mitochondrial nor ER [11]. Gimap proteins may therefore have similar function, but different subcellular locations. At this time, there is a paucity of information as to the expression of the *Gimap* genes in specific cell types.

The fact that the *Gimap* genes are located together in a tight cluster on RNO4 (and in conserved synteny with many other species), combined with their sequence similarities, suggests the possibility that the proteins carry out similar function. While there is sufficient evidence to support the frameshift mutation in *Gimap5* as the cause of lymphopenia, we could not exclude that either *Gimap1* or *Gimap3* play a role, as they are located within the lymphopenia critical interval between D4Rhw6 and IIsnp3 as well as within the PAC used in the transgenic rescue of lymphopenia [7]. In addition, it is possible that the remaining *Gimap* family members outside the lymphopenia critical interval play a role in T1D development. In order to substantiate the frameshift mutation in *Gimap5* and the subsequent protein null allele as the cause of lymphopenia as well as explore a possible contribution by other *Gimap* family members, we sequenced DR.$^{+/+}$ and DR.$^{lyp/lyp}$ cDNA from rat thymus. In addition, we examined *Gimap* gene expression across multiple tissues and quantified mRNA expression of all annotated and putative *Gimap* genes in DR.$^{+/+}$ and DR.$^{lyp/lyp}$ rat thymus, spleen, and mesenteric lymph node (MLN).

2. Materials and Methods

2.1. DR.lyp Congenic Rats. The DR.*lyp* (BBDR.BBDP-(*D4Rhw17-SS99306861*) (*D4Rhw11-D4Rhw10*)/*Rhw*) congenic rat line was derived from animals with two independent recombination events developed from our previously described introgression of the lymphopenia locus by cyclic cross-intercross breeding of BBDP with BBDR rats [17]. The first recombination event was flanked

by simple sequence length polymorphism (SSLP) marker D4Rhw11 (76.81 Mb) and the second flanked by SSLP marker D4Rhw10 (77.81 Mb) [4]. Thus, the DP DNA in the DR.*lyp* rat line encompasses the *lyp* critical interval from D4Rhw6 (76.83 Mb) to IIsnp3 (77.16 Mb) [2]. In addition, the DR.*lyp* congenic rat line used in the present study also contains BBDP DNA at D4Rat102 (66.22 Mb) and D4Rat26 (69.18 Mb). The DR.*lyp* congenic rat line is kept in sister-brother breeding and produces Mendelian proportions of the DR.$^{lyp/lyp}$ (25%), DR.$^{lyp/+}$ (50%), and DR.$^{+/+}$ (25%) genotypes. DR.$^{lyp/lyp}$ are 100% lymphopenic and 100% diabetic.

2.2. Housing. Rats were housed in a specific pathogen—free facility at the University of Washington, Seattle, Washington, on a 12-hour light/dark cycle with 24-hour access to food (Harlan Teklad, Madison, Wis, USA) and water. All protocols were approved by the institutional animal use and care committee of the University of Washington, Seattle, Wash, USA. The University of Washington Rodent Health Monitoring Program was used to track infectious agents via a quarterly sentinel monitoring system. Excluded infectious agents are listed at http://depts.washington.edu/compmed/rodenthealth/index.html#excluded.

2.3. RNA Isolation. Thymus, spleen, and mesenteric lymph nodes were homogenized from 45–78-day-old DR.*lyp* rats (7 male, 8 female) immediately after dissection in RNA lysis solution (Stratagene, La Jolla, CA or Qiagen, Valencia, Calif, USA) either with a pestle (Kontes, Vineland, NJ, USA) and, if viscous, passed through a 20 gauge needle or a Kinematica Polytron PT 10/35 (Brinkmann, Westbury, NY, USA). Bone marrow was obtained by flushing the femora and tibia with Dulbecco's modified medium (Life Technologies, Grand Island, NY, USA). Nucleated cells were separated with lympholyte-rat (1.094 g/cm³, Cedarlane Lab, Ontario, Canada) according to the manufacturer's protocol. Total RNA was isolated using either RNeasy (Qiagen) or Absolutely RNA Miniprep Kit (Stratagene) followed by treatment with DNase. PolyA+ RNA was isolated with Oligotex Direct mRNA Midi/Maxi Kit, (Qiagen). Total RNA was quantitated with RiboGreen (Stratagene).

2.4. cDNA Cloning and Sequencing. cDNA synthesis was performed using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, Calif, USA) according to the manufacturer's recommendations. PCR products were amplified from thymus cDNA as follows: PCR products were generated by using either Herculase (Stratagene) or Roche Taq polymerase (Roche Diagnostics, Indianapolis, Ind, USA). Reactions with Herculase were 25 μL, consisting of 100 ng cDNA, 0.5 μL of Herculase polymerase, 2.5 μL of the supplied buffer, 0.5 μL of a mix of 10 mM each dNTP, and 2 μL each 5 μM primer. Amplification was carried out in a PTC-200 Peltier Thermal Cycler (Bio-Rad, Hercules, Calif, USA) with the following conditions: 95°C for 3 minutes, 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 6 minutes, and

TABLE 1: Probes and primers used in qRT-PCR.

Primer name	Primer sequence 5′ to 3′
Gimap8-f	CCAGGAGACCCAGGTGAAAG
Gimap8-r	AGTTGAATGCTCATCATAGCTCCTT
Gimap8-p	6FAM-TCTGTTGACAATAGCCAATGATCTCA-BHQ1
Gimap9-f	AGGAACGGCAGAGCCTACTTT
Gimap9-r	CCACTAGACATTGGTTCAGCTTCTTA
Gimap9-p	6FAM-CTGACAGGATATATAAGGACA-MGBQ
Gimap4-f	AACATGCCGTACAGAGCTCACA
Gimap4-r	AGTGGCACCATTAGAAGGCAAA
Gimap4-p	6FAM-CCATGACACACCCACTCCAACAGGG-BHQ1
Gimap6-f	TGGATGCTCTGGATGTTGCA
Gimap6-r	TCCTGCTCATCCCCTTGTG
Gimap6-p	6FAM-TTGTTGAAGCCACAATGGCGTCTCTCA-BHQ1
Gimap7-f	GGACTCAGTGTCAGGCTCCAA
Gimap7-r	CGGGAGGACAGGCTAGCATA
Gimap7-p	6FAM-CTGGATCACACTTGGCGCTCAGCTC-BHQ1
Gimap1-f	AGAGGCGGACCAGGTTCCTA
Gimap1-r	CCTCCAGCCCTGCCTGTAG
Gimap1-p	6FAM-TTCTGCCATCTCCACAGCCCA-BHQ1
Gimap5-f	CATGTTAGGGAAGCTCAGTC
Gimap5-r	GAAGGGTTCTACTGTGTCTCA
Gimap5-p	6FAM-TTTCACTATCATTTGACTCCTGTGCA-BHQ1
Gimap3-f	CCACAGGGAGTGTAGACCTTGAA
Gimap3-r	CTGCTGTTTCCGAATCCAGTTT
Gimap3-p	6FAM-ATCCTCCAGCGTCCAC-MGBQ
Lr8-f	GCCTCTGGTTGTGCCTTCTG
Lr8-r	CCCTGTCCCATCTCATGGAT
Lr8-p	6FAM-CCCACTCCAGCCAAAATTGCCACA-BHQ1
Cyc-f	CACCGTGTTCTTCGACAT
Cyc-r	TTTCTGCTGTCTTTGGAACT
Cyc-p	HEX-CTGCTTCGAGCTGTTTGCAGAC-BHQ1

Probes and primers were designed to bind near the 3′ end of the transcripts. f is forward primer, r is reverse primer, p is probe, 6FAM is 6-carboxyfluorescein, HEX is hexachlorofluorescein, and BHQ1 is black hole quencher 1.

a final step of 72°C for 7 minutes. Reactions with Roche polymerase were 20 μL, consisting of 100 ng cDNA, 0.1 μL Roche Taq polymerase, 2.0 μL of the supplied buffer, 0.5 μL of a mix of 10 mM each dNTP, and 2 μL each 5 μM primer. Reactions were carried out with the following conditions: 94°C for 3 minutes, 35 cycles of 94°C for 45 seconds, 60°C for 45 seconds, 72°C for 2 minutes, and a final step of 72°C for 7 minutes. PCR products were cloned into pCRII with the TOPO-TA cloning kit (Invitrogen), sequenced using ABI BigDye v3.1 (Applied Biosystems, Foster City, Calif, USA), and analyzed on an ABI 3730XL sequencer (Applied Biosystems) at the University of Washington Biochemistry Sequencing Core in Seattle, WA. 5′ and 3′ RACE (5′ and 3′, Rapid Amplification of cDNA Ends) was carried out with a Marathon cDNA Amplification kit (K1802-1, Clontech, Palo Alto, Calif, USA) using the protocol provided. Plasmids were transformed into XL1Blue (Stratagene) by electroporation of Top10 cells (Invitrogen) according to the protocol supplied with the cells. Plasmids were purified by using GenElute

Plasmid Maxiprep Kit (Sigma, St. Louis, Mo, USA) or Plasmid Maxi Kit (Qiagen). GenBank accession numbers of the cloned genes and of RACE products are DQ125335–DQ125353.

2.5. Quantitative RT-PCR. RNA was collected from whole tissue, isolated using a Qiaqen RNeasy minikit (Valencia, Calif, USA), and aliquoted to minimize degradation from freezing/thawing. Quantitative real time polymerase chain reaction (qRT-PCR) was performed on anMx4000 Multiplex QPCR System (Stratagene) in duplex reactions with rat cyclophilin (NM_017101) as an internal control. Samples were run in triplicate using 100 ng of total RNA or 5 ng of polyA+ RNA. Twenty-five μL reactions were run using a Brilliant Single-Step qRT-PCR Kit (2.5 μL 10x core RT-PCR buffer, 5.5 mM MgCl$_2$, 300 nM each primer, 200 nM each probe, 0.3 mM dNTP, 75 nM passive reference dye, 1.6 units Stratascript RT, 2 units SureStart Taq DNA-polymerase). The
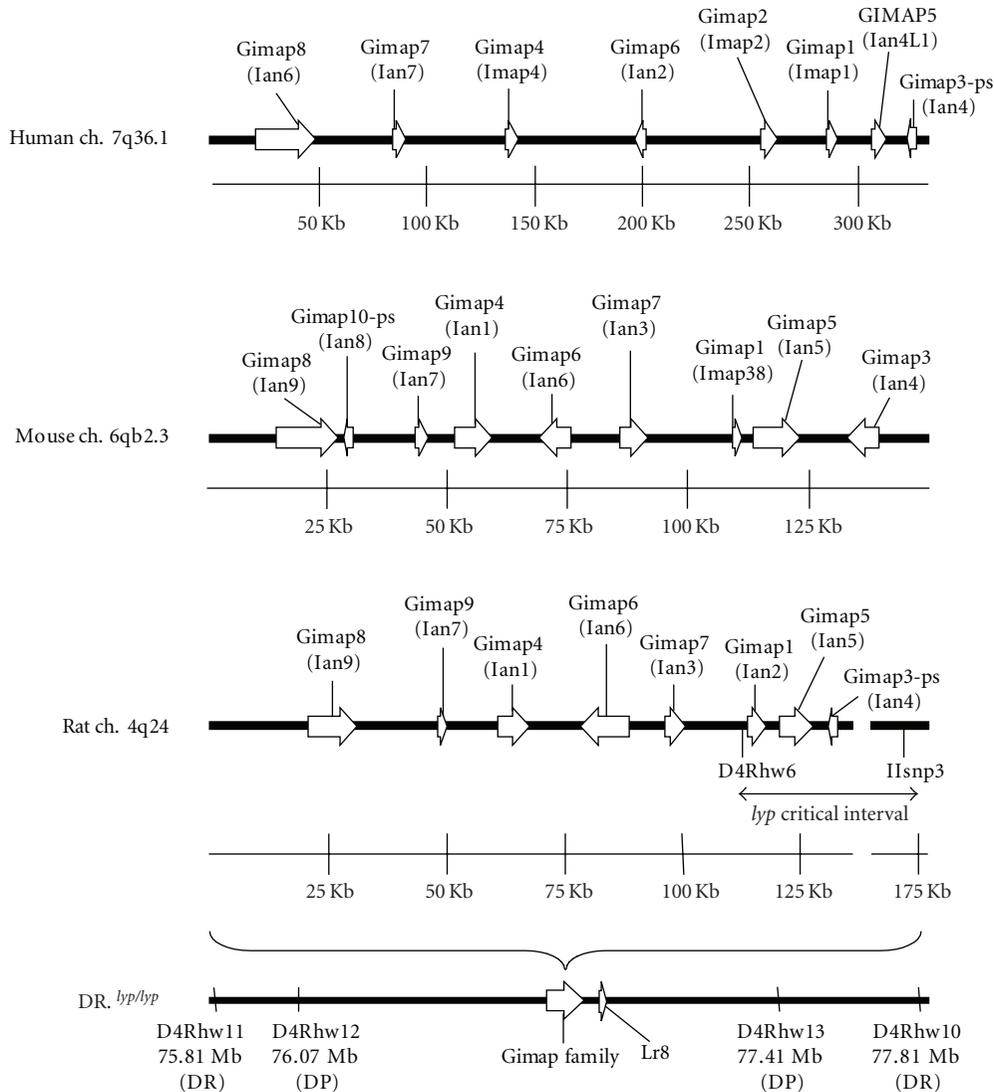
FIGURE 1: *Expanded map of Gimap interval in human, mouse, and rat. Gimap* family orthology in human, mouse, and rat is shown along with an expanded map of the 2 Mb of DP DNA in the congenic DR.*lyp* rat line. The 33 Kb lymphopenia critical interval is indicated between the SSLP markers D4Rhw6 and IIsnp3. *Ian* aliases are in parentheses underneath the corresponding *Gimap* name.

PCR cycling conditions were as follows: 45°C for 30 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 30 seconds, 60°C for 1 minute. Probes were positioned in the 3′ regions of the transcripts where there is more variation between the different *Gimap* genes and subjected to BLAST alignment to ensure specificity. The primers and probes used for each gene are listed in Table 1. Primers were obtained from Integrated DNA Technologies (Coralville, Iowa, USA) or Qiagen Operon (Valencia, Calif, USA). Fluorescently labeled probes were obtained from Integrated DNA Technologies. Representative qRT-PCR products for each gene, from each tissue, were run on an agarose gel to check for primer pair binding specificity. Each assay was also optimized and validated with serial dilutions of RNA to produce a standard curve that was then translated into a reaction efficiency, or specificity, of each *Gimap* gene assay. Results from each assay were validated and normalized against cyclophilin. The

standard curves, multiplexed with cyclophilin, showed the following reaction efficiencies: *Gimap8*: 90% ± 2 SD, *Gimap7*: 87% ± 4 SD, *Gimap4*: 92% ± 2 SD, *Gimap6*: 94% ± 3 SD, *Gimap9*: 98% ± 7 SD, *Gimap1*: 100% ± 11 SD, *Gimap5*: 88% ± 6 SD, *Lr8*: 93% ± 4 SD, and cyclophilin: 90% ± 6 SD.

2.6. *Statistical Analysis.* Three DR.$^{+/+}$ rats from different litters were used to determine the expression of *Gimap* genes in mesenteric lymph node, thymus, spleen, bone marrow, and kidney. To compare *Gimap* and *Lr8* gene expression across multiple tissues, data was first normalized to cyclophilin then scaled and expressed as a percentage of DR.$^{+/+}$ *Gimap5* mesenteric lymph node (MLN), the highest expressing gene overall. For analysis of *Gimap* expression in DR.$^{lyp/lyp}$, DR.$^{lyp/+}$, and DR.$^{+/+}$ rat thymus, spleen, and mesenteric lymph node, 15 rats (5 rats per genotype) were

TABLE 2: *Gimap* family thymus cDNA sequencing in DR.$^{+/+}$ and DR.$^{lyp/lyp}$ rats.

Gene name	Location (bp)	RefSeq identifier	mRNA position	DR.$^{+/+}$	DR.$^{lyp/lyp}$	F344	A.A Change	Genbank accession #
Gimap8	76738163	NM_001033923	−96	C	T		5′ UTR	DQ125335-36
			−11	T	C		5′ UTR	
Gimap9	76765555	NM_001008398	928	C	T		3′ UTR	DQ125337-38
D4Rhw2	**76.77**							
Gimap4	76777679	NM_173153	216	A	G	A	G 72 G (Synonymous change)	DQ125339-40
			510	G	A	G	T 170 T (Synonymous change)	
			618	G	A	G	L 206 L (Synonymous change)	
			922-923	TA	—	—	YLN* 308 LELIIKAWEIASFIFNQFMRD*	
Gimap6	76794903	NM_001011968	No SNPs					DQ125342-43
Gimap7	76812445	NM_001024328	603	G	A		V 201 V (Synonymous change)	DQ125348-49
D4Rhw6	**76.82**							
Gimap1	76829536	NM_001034849	752	T	C		M 251 T IFESKIQNQDMDKDIGNCY…	DQ125350-51
Gimap5	76836521	NM_145680	252	C	—		85 SSSQRSRTKTWTRTLGTAT*	DQ125352-53
			523	C	T		L 175 -	
IIsnp3	**77.16**							

mRNA position is relative to the ATG start site. UTR is untranslated region.

used from 5 litters consisting of 1 rat genotype from each litter. Mesenteric lymph node data for 1 litter was missing leaving 12 rats from 4 litters for analysis. Comparisons from bone marrow and kidney are not shown due to very low expression and high error in these tissues. Pairwise comparisons of the individual ratios were carried out using linear mixed effects models in S-PLUS (Insightful Corp., Seattle, Wash, USA) with a random intercept for each litter. A conditional F-test was implemented to test the significance of terms in the fixed effects models. A two-tail test with a P-value <.05 was judged as significant.

2.7. Bioinformatics. Predicted protein sequences were aligned using T-coffee (http://www.ch.embnet.org/software/TCoffee.html). Structure and topology of proteins were defined using HMMTOP (http://www.ensim.hu./hmmtop/index.html) or Protein Predict (http://cubic.bioc.columbia.edu/pp/). Subcellular locations were predicted using PSORT (http://psort.nibb.ac.jp/form2.html). The nomenclature used in this paper follows the official names determined by the rat nomenclature committee (Lois J. Maltais, Mouse Genome Database (MGD), Mouse Genome Informatics Web Site, The Jackson Laboratory, Bar Harbor, Maine, http://www.informatics.jax.org/) and is different from previous publications [2, 3, 18, 19].

3. Results

3.1. cDNA Cloning and Sequencing of Gimap8, Gimap9, Gimap4, Gimap6, and Gimap7 in DR.$^{+/+}$ and DR.$^{lyp/lyp}$ Rats.

Gimap8, Gimap9, Gimap4, Gimap6, and *Gimap7* (in the order as they appear on the chromosome) are located outside the lymphopenia critical interval (Figure 1). Sequence analysis of thymus cDNA encoding *Gimap4* showed three single nucleotide polymorphisms (SNPs) at positions 216, 510, and 618, relative to the ATG start site, and two nucleotides deleted at position 922-923 in DR.$^{lyp/lyp}$ rats as compared to DR.$^{+/+}$ rats (Table 2). The first three base pair substitutions resulted in synonymous amino acid changes in the hypothetical protein sequence, while the deletion resulted in a frameshift mutation in the last three predicted amino acid residues and eliminated the normal stop codon at position 311. 3′ RACE from DR.$^{lyp/lyp}$ thymus cDNA showed that the reading frame continued for other 21 amino acids before generating a new stop codon (Table 2). This same frameshift mutation was also identified in F344/Rhw (nonlymphopenic) rats, which were used in the positional cloning of lymphopenia (Table 2). One nucleotide substitution was identified in *Gimap7* at position 603, relative to the ATG start site, between DR.$^{+/+}$ and DR.$^{lyp/lyp}$ that resulted in a synonymous amino acid change in the hypothetical protein sequence (Table 2). No SNPs were found in the coding sequences of *Gimap6*, *Gimap8*, and *Gimap9*.

3.2. cDNA Cloning and Sequencing of Gimap1 and Gimap3 in DR.$^{+/+}$ and DR.$^{lyp/lyp}$ Rats. *Gimap1* and *Gimap3* are located inside the lymphopenia critical interval (Figure 1). Sequence analysis of thymic cDNA showed a single SNP in the coding sequence of *Gimap1* at nucleotide position 752, relative to the ATG start site, that produced an amino

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                                             GTP                             GTP                       GTP                                Conserved
Gimap8A (1–250)   . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . M  ATSSHQGAAA  GSQME.HRLC  ETSIGQGERP  RASRGQE...  .SNFKQSQG.
Gimap8B (251–465) . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . .GVQGCGT  GVTYKGDNLC  GS........  KKRQPQI...  .TGPGWDRD.
Gimap8C (466–688) . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . IREK
Gimap9            . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . .MADYE.
Gimap4            . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  MEA.QYSGVG  SIPENSRSSH  .ELGIQDQGS
Gimap6            MNWLYSKTLG  SIGSCCIDTL  PWPFHSFFQR  NLLALPGEPG  NPLESSATES  GKQSRSCLSA  SPVME.EEGC  EHSLQKNPTR  QLPLDPGQEL  TKDLKEKKLT
Gimap7            . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . .MAELD.
Gimap1            . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . .MGGR  KMVRD.EEGA  YGSEDDSR..  ..........  ......APQE
Gimap5            . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  MEGLQKSTYG  TIVEGQETYS  VE.......D

Gimap8A (1–250)   TSTLRLLLLG  KQGAGKSATG  NTILGKAVFE  SRFSH.HMVT  KRCQSESVSV  RGKQVIVIDT  PDLFSSLGC.  ..PEVQQQNL  RQCL.DLL.A  DPYVLLLVTP
Gimap8B (251–465) TPELRVLLMG  KRGVGKSAAG  NSILGKQVFK  TQFSEKQRVT  EAFASHSRLW  NQKKFLIIDS  PEISSWKLD.  ..ESDVKE..  .....HTF.P  GPHAFLLVTP
Gimap8C (466–688) .ELLNIIILG  RSGVGKSATG  NTILGRPAFV  SQLRA.QPVT  SRSQSGRRTL  DWQDIVVVDT  PSLNQMSGTE  KNPAQLKKEI  KQCLLQNCEE  GMKVFVLVFQ
Gimap9            DSEMRIILVG  KTGNGKSATA  NTILGKCQFD  SKICA.YAVT  KTCQRASRRW  KGKDLVVVDT  PGLFDTKES.  ..LKTTCSEI  SRCVLYSC.P  GPHAIILVLR
Gimap4            .PQLRIVLLG  KTGAGKSSTG  NSILGRKAFL  SGICA.KS1T  KVCEKGVSIW  DGKELVVVDT  PGIFDTEVP.  ..DADTQKEI  TRCVALTS.P  GPHALLLVTP
Gimap6            PKRLQLLLVG  KTGSGKSATG  NSILGRQVFE  SKISA.RPVT  MAFQKGSREL  EGKELEVIDT  PDILSPQNQ.  ..PEATAKKI  ..CDILAS.P  GPHAVLLVIQ
Gimap7            DDSLRIVLVG  KTGSGKSATA  NTILGQKIFT  SRIAP.HAVT  QTCQKASRRW  KERDLLVVDT  PGLFDTKVN.  ..LETTSIEI  SRCVLQSC.P  GPHAIILVLQ
Gimap1            .PHLRLILVG  RTGTGKSATG  NSILGQKCFL  SRLGA.VPVT  RSCTLASRRW  AGRLVEVVDT  PDIFSSETPR  SDPG..CVEA  ARCFVLSA.P  GPHALLLVTQ
Gimap5            SGLLRILLVG  KSGCGKSATG  NSILRRPAFE  SRLRG.QSVT  RTSQAEMGTW  EGRSFLVVDT  PPIFESKIQ.  ..NQDMDKDI  GNCYLMCA.P  GPHVLLLVTQ

                  Box
Gimap8A (1–250)   IGHSTEEDKK  TIEGIQGVFG  PQAYRHMIVV  FTREDELGED  TLQNHIESKK  .Y.LKKLIEN  IGSQRCCAFN  NKADKKQQEL  QVSQFLDAIE  FLMMESPGTY
Gimap8B (251–465) LGSSLKSGDS  VFSIIKRIFG  EKFIKFTIIL  FTRKEDFEGQ  DLDTFTKEND  ..ALCNLIQI  F.EGRYAVFN  YRATVEEEQS  QVGKLLSQIE  SVVQHHNNKP
Gimap8C (466–688) LGRFTQEDEA  VVEQLEASFE  ENIMKYMIVL  FTRKEDLGDG  DLYDFTNNTK  NKVLKRIFKK  C.KGRVCAFN  NKETGEDQET  QVKALLTIAN  DLKRSYD.EH
Gimap9            LDRYTEEEQK  TVALIQGLFG  EAALKYMIIL  FTHKEDLEGQ  SLDNFVDDAG  .EKLNNIVSQ  C.GKRYLAFN  NKAAEAEQEN  QVQQLIDLIE  EMVARNGRAY
Gimap4            LGRYTVEEHK  ATRKLLSMFE  KKARRFMILL  LTRKDDLEDT  DIHEYLE.TA  PEVLQELIYE  F.RNRYCLFN  NKASGAEQEE  QKRQLLTLVQ  SMVRENGGKY
Gimap6            VGRYTTEDQE  AARCLQEIFG  NGILAYTILV  FTRKEELAEG  SLEEYIKENN  NKTLDALDVA  C.ERRHCGFN  NRAQGDEQEA  XLQKLMEEIE  SILWENEGHC
Gimap7            LNRFTIEEQE  TVTRIKAIFG  KAVMKYLIIL  FTRKDELEDQ  NLNDFIEDSD  .TNLKSIIKE  C.DSRYLAIN  NKAEGAGEGM  QVQELMGFVE  SLVRSNGGLY
Gimap1            LGRFTTQDSQ  ALAAVKRMFG  KQVMARTVVV  FTRKEDLAGD  SLQDYVRCTD  NRALRELVAE  C.GDRVCALN  NRATGSESEA  QVEQLLDLVA  CLVREHRGTH
Gimap5            LGRYTVEDAM  AVRMVKQIFG  VGVMRYMIVL  FTHKEDLADE  SLEEFVTHTG  NLDLHRLVQE  C.GRRYCAFN  NKASGEEQQG  QLAELMALVR  RLEQEHEGSF

Gimap8A (1–250)   FEPLKTENS.  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .
Gimap8B (251–465) CV......  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .
Gimap8C (466–688) STSWMDQLKS  AVG......  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . . . . . . . .  . . . .QITTV  .....FK...
Gimap9            FSDRIYKDID  KKLNQCLVLD  KETYTQQLLS  EIQRIETECA  ..NKSEKEKE  AQIVSARRNY  DETIRKLKEK  AEENVFTYIF  .....QKITE  ILSKLWDIL.
Gimap4            FTNKMYESAE  GVIQKQTWKK  KEFYREELER  ERARIR....  ......REYE  AEIQDLRDEL  ERERRRARME  REFNENELIF  AERQQNARRE  VENTSMIYLN
Gimap6            YTMELPNVSS  KTL......
Gimap7            FSDPIYKYAE  QRLKKQVGIL  REIYTDVLEK  EIRIVEEECG  LGKLSTQEGE  EKIQAIREKY  NLKIGNLREA  AEKNIFTWIV  .....EEVKK  VLLKIWHFFS
Gimap1            YSNEVYELVQ  ATRCADPQDQ  LTKVAEMVV.  .....ARMQ.  ......RRTR  FLAGLWRWQK  SYRQ......  ..GWRGLMIF  .....VGAAL  LICLLFYRKV
Gimap5            HSNDLFVYTQ  VFLRGGYSEH  QEPYKFYLTK  VRQEVEKQK.  ......RELE  EQEGSWMAKM  LCRVTSCLDW  HIAVSVLLIV  .....LGLTL  LITLINMYIG

Gimap8A (1–250)   .......
Gimap8B (251–465) .......
Gimap8C (466–688) .......
Gimap9            .......
Gimap4            .......
Gimap6            .......
Gimap7            .......
Gimap1            PGLWGPE
Gimap5            RWK....
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Figure 2: *Alignment of predicted Gimap protein sequences in the DR.$^{+/+}$ rat.* T-coffee predicted protein alignment from cloned cDNA is shown. *Gimap8* is divided into three separate sequences (with the amino acid numbers indicated by each sequence). Shading indicates the GTP binding domain consensus regions (GTP) and the conserved domain (*Conserved Box*). The HMMTOP predicted transmembrane domain sequences for *Gimap1* and *Gimap5* and the coiled coil domains for *Gimap4* and *Gimap9* are underlined.

acid change in DR.$^{lyp/lyp}$ rats as compared to the DR.$^{+/+}$ (Table 2). The SNP produced a methionine (M) to threonine (T) substitution at amino acid 251, which is located near the C-terminus and is not in any of the predicted GTP binding domains. Sorting intolerant from tolerant (SIFT) analysis (http://blocks.fhcrc.org/sift/SIFT.html) predicted the T substitution to be tolerated at this position and did not predict to affect protein function.

Gimap3 is not annotated in the rat genome sequence. Genomic sequencing of the putative ortholog of mouse *Gimap3* from base pair positions 76,846,091 to 76,852,162 on RNO 4 (the orthologous DNA interval to mouse *Gimap3*) in DR.$^{+/+}$ and DR.$^{lyp/lyp}$ rats revealed repetitive single or dinucleotide repeats throughout the region that likely resulted in early termination of the sequencing reactions. As such, no specific PCR products could be generated. Attempts were also made to amplify *Gimap3* from DR.$^{+/+}$ and DR.$^{lyp/lyp}$ rat thymus cDNA; however, again, no specific PCR products were obtained. Comparative analysis of the Brown Norway (BN/Hsdmcwi) database sequence, available at the University of California Santa Cruz (UCSC; Nov. 2004 assembly), with the mouse *Gimap3* database sequence (UCSC) failed to establish an open reading frame. The multiple repetitive elements added additional difficulty in locating potential exons or transcripts. No rat EST evidence could be found in the region orthologous to mouse *Gimap3* and in human; *GIMAP3* is annotated as a pseudogene. Lastly, no evidence of a *Gimap3* transcript was found in northern
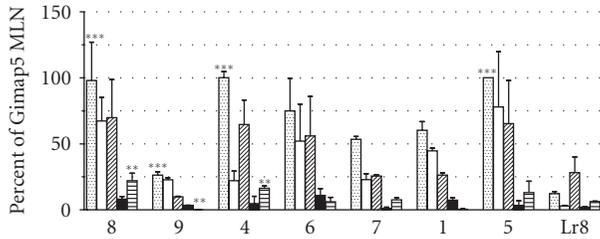
FIGURE 3: *Tissue specific Gimap expression.* The mean ± standard deviation is shown for DR.$^{+/+}$ ($n = 3$) *Gimap* gene expression. To compare *Gimap* gene expression across multiple tissues, data was first normalized to cyclophilin then scaled and expressed as a percentage of DR.$^{+/+}$ *Gimap5* mesenteric lymph node (MLN), the highest expressing gene overall. Genes appear in the order at which they appear on rat chromosome 4. Tissues appear in the following order per gene: MLN (dots), thymus (white), spleen (hash marks), bone marrow (black), and kidney (stripes). Significance is represented as follows: ∗∗∗ is $P < .0001$ and ∗∗ is $P < .001$.

blots of DR.$^{+/+}$ and DR.$^{lyp/lyp}$ or from qRT-PCR of DR.$^{+/+}$ rat thymus or spleen (data not shown). Therefore, *Gimap3* is likely a pseudogene in rat.

3.3. Predicted Protein Alignment. Alignment of the Gimap family predicted protein sequences in the DR.$^{+/+}$ rat (Figure 2) showed predicted GTP binding domains and conserved box characteristics for all of the Gimap proteins with the most divergent regions located near the C-terminal ends. Gimap1 and Gimap5 are predicted to contain transmembrane domains while Gimap4 and Gimap9 are predicted to contain coiled coil domains. Gimap8, Gimap7, and Gimap6 are predicted to have neither transmembrane nor coiled coil domains. Gimap8 was larger than the other Gimap proteins, containing 688 amino acids and three repeated GTP binding domains (Figure 2).

3.4. Gimap Expression Pattern across Multiple Tissues. The relative expression levels of the *Gimap* genes in mesenteric lymph node, thymus, spleen, bone marrow, and kidney were determined in the DR.$^{+/+}$ rat (Figure 3). All of the *Gimap* genes expressed more robustly in the mesenteric lymph nodes, thymus, and spleen as compared to bone marrow and kidney ($P < .0001$). In the mesenteric lymph node, *Gimap4*, *Gimap5*, and *Gimap8* were expressed significantly higher than *Gimap9* ($P < .0001$) while in kidney, *Gimap4* and *Gimap8* were expressed significantly higher than *Gimap1* and *Gimap9* ($P < .001$) (Figure 3). No significant expression differences were detected between any of the *Gimap* genes (*Gimap8, 9, 4, 6, 7, 1*, and *5*) in thymus, spleen, and bone marrow. Overall, *Lr8*, a gene unassociated with the *Gimap* family but also within the 2 Mb of DP DNA in the congenic DR.$^{lyp/lyp}$ rat line, expressed predominantly in the spleen, an expression pattern unique relative to the *Gimap* family.

3.5. Gimap Expression in DR.$^{lyp/lyp}$ and DR.$^{+/+}$ Thymus, Spleen, and Mesenteric Lymph Node. In thymus, expression of *Gimap4, Gimap9, Gimap1*, and *Gimap5* was significantly

decreased in DR.$^{lyp/lyp}$ rats as compared to DR.$^{+/+}$ (Figure 4). In contrast, *Gimap7* expression in thymus was higher in DR.$^{lyp/lyp}$ as compared to DR.$^{+/+}$ while *Gimap8, Gimap6*, and *Lr8* showed no differential expression. Expression of all of the *Gimap* genes (*8, 9, 4, 6, 7, 1*, and *5*) was reduced in DR.$^{lyp/lyp}$ rat spleen and mesenteric lymph node as compared to DR.$^{+/+}$ (Figure 4). We observed the same expression pattern whether the data were normalized to cyclophilin or to total RNA (data not shown). Data from bone marrow and kidney is not shown due to the very low expression in these tissues. The low expression observed in these tissues is not due to RNA degradation, but rather to the low mRNA levels relative to cyclophilin levels.

4. Discussion

While the frameshift mutation in *Gimap5* is likely necessary and sufficient for lymphopenia, the possibility remains that additional mutation(s) in the *Gimap* family may contribute to the development of lymphopenia, spontaneous T1D, or both in the DR.$^{lyp/lyp}$ rat. Aside from *Gimap5*, only *Gimap1* and *Gimap3* could potentially play a role in the development of lymphopenia, as they are the only remaining genes located within the 33 Mb interval critical for lymphopenia between D4Rhw6 and IIsnp3. The methionine to threonine base pair substitution at amino acid position 251 in *Gimap1* is not located in any of the predicted GTP binding domains and SIFT analysis predicted that the mutation is not likely to alter normal Gimap1 protein function. Furthermore, the DR amino acid at position 251 is not conserved across species; human GIMAP1 has a valine at this same position [3]. Lastly, no *Gimap3* transcript could be found and no open reading frame could be identified. We suspect that this region of the rat genome does not code for a protein, which is similar to the human GIMAP3 pseudogene [3] and unlike mouse *Gimap3* [15]. Therefore, the sequence analysis of *Gimap1* and *Gimap3* supports the hypothesis that *Gimap5* is the cause of lymphopenia in the DR.$^{lyp/lyp}$ rat. In addition, the sequence data from all of the remaining *Gimap* family members suggest that these genes are not likely to play a role in the onset of T1D diabetes. We cannot however exclude the possibility that there are mutations outside of the coding regions, such as in transcription factor binding sites or other regulatory sites, that play a role in the regulation of *Gimap* gene expression and/or the onset of T1D in the DR.$^{lyp/lyp}$ rat.

While we can exclude the involvement of the members of the *Gimap* family proximal to D4Rhw6 (*Gimap8, Gimap9, Gimap4, Gimap6*, and *Gimap7*) in the development of lymphopenia, we cannot exclude that they may play a role in the development of T1D. Coding sequence analysis of these family members revealed that only *Gimap4* had genetic differences, specifically a two-base pair deletion, that would result in a nonsynonymous amino acid change between the nondiabetic DR.$^{+/+}$ and the diabetes susceptible DR.$^{lyp/lyp}$ (Table 2). Although the effect of this variation is unknown, we did discover this same deletion in the nonlymphopenic, diabetes resistant F344 rat. F344 DNA introgressed through this interval on the DR.$^{lyp/lyp}$ background protects from
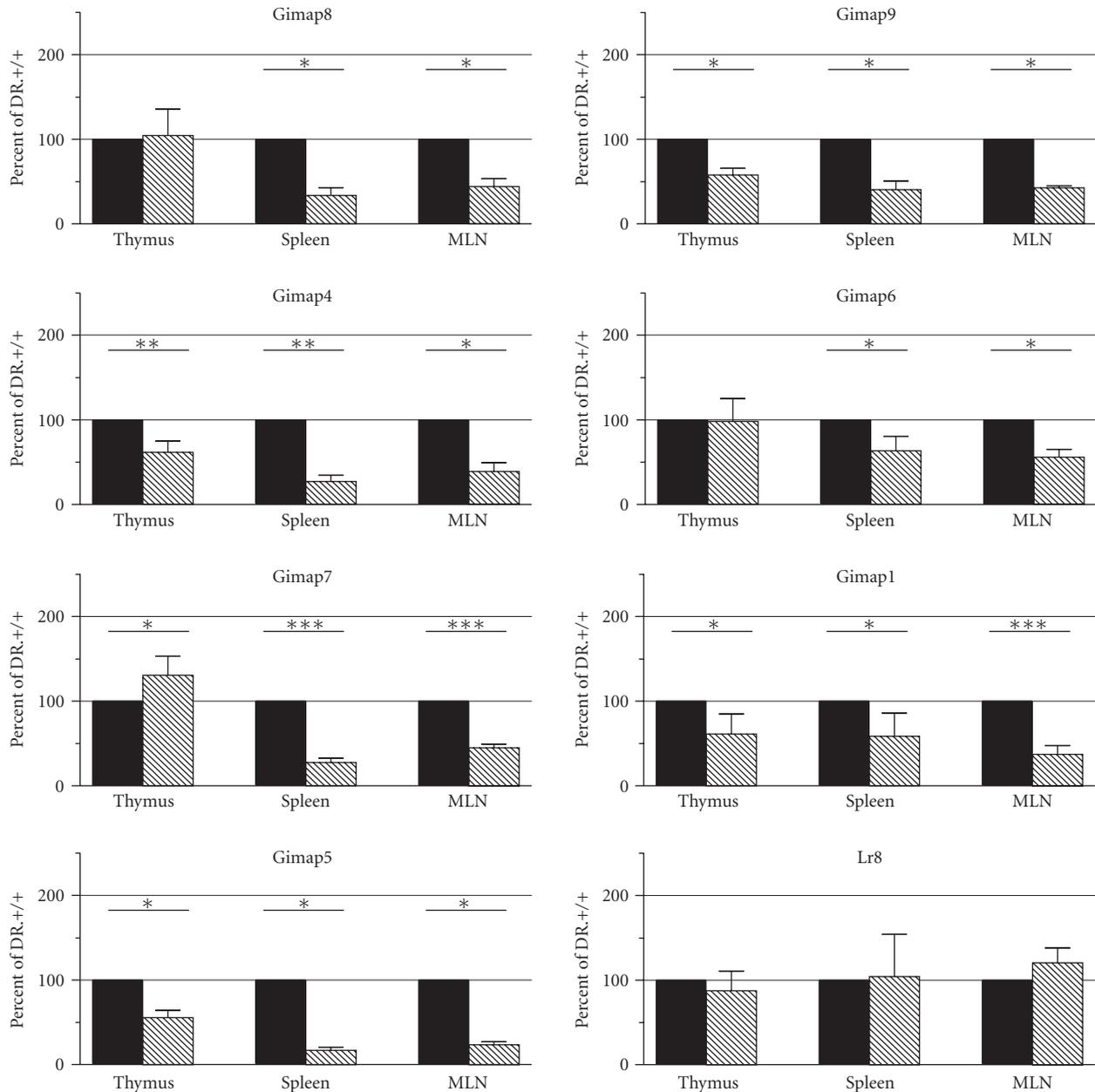
FIGURE 4: *Gimap gene expression in the DR.*$^{+/+}$ *and DR.*$^{lyp/lyp}$ *rats.* The mean ± standard deviation is shown for DR.$^{+/+}$ and DR.$^{lyp/lyp}$ rat *Gimap* gene expression in thymus ($n = 5$), spleen ($n = 5$) and mesenteric lymph node (MLN) ($n = 4$) after normalization to cyclophilin. Black columns represent DR.$^{+/+}$ and grey hatched columns represent DR.$^{lyp/lyp}$. Data is expressed as a percentage of DR.$^{+/+}$. Significant differences are follows; ∗ for $P < .05$, ∗∗ for $P < .01$, ∗∗∗ for $P < .001$. Genes appear in the order at which they appear on rat chromosome 4. The average *Gimap* expression in DR.$^{+/+}$ rat bone marrow and kidney s is shown in Figure 3.

onset of T1D [4] suggesting that the deletion mutation in *Gimap4* is not deleterious. In addition, the predicted protein sequences of both human (AK001972) and mouse (NP_778155.2) Gimap4 show that the 23 C-terminal amino acids are similar to those of DR.$^{lyp/lyp}$ (data not shown). It is therefore unlikely that the *Gimap4* two-base pair deletion mutation in the DR.$^{lyp/lyp}$ rat is functionally relevant to development of T1D or lymphopenia, rather it is likely an additional natural isoform [20].

All of the *Gimap* genes were predominantly expressed in organs of the immune system: mesenteric lymph node, thymus, and spleen, consistent with previous findings of a role of the *Gimap* gene family in lymphocyte development [21]. Interestingly, there was an overall reduction in expression of all seven *Gimap* genes in DR.$^{lyp/lyp}$ rat spleen and mesenteric lymph node and four (*Gimap4*, *Gimap9*, *Gimap1*, and *Gimap5*) of the seven genes in DR.$^{lyp/lyp}$ rat thymus. In contrast, *Lr8*, a gene unrelated to the *Gimap* family located

69 Kb downstream of *Gimap5*, (Figure 1) [22, 23] showed no differential expression between DR.$^{+/+}$ and DR.$^{lyp/lyp}$. It is not clear why *Gimap5* transcript levels are reduced as the single cytosine residue deletion results in a frameshift mutation and a premature truncation in the protein. One hypothesis is that during protein synthesis, the incomplete (truncated) protein may destabilize the RNA/protein complexes and cause mRNA degradation [24, 25]. Another hypothesis for reduction in DR.$^{lyp/lyp}$ rat *Gimap* gene transcripts is a difference in organ composition from those of DR.$^{+/+}$ rats. Reduced T cells numbers in DR.$^{lyp/lyp}$ rats could lead to a difference in cellular composition and may explain the lower observed expression of all of the *Gimap* genes. As the function of Gimap proteins remains rather poorly defined, it would be a useful addendum if in vitro knockdown experiments were designed to test the functional changes that might derive from reduced expression of *Gimap5*. These types of experiments could determine if the mutation is significant in functional terms or if the altered expression is the most critical feature.

The *Gimap* gene family is conserved throughout evolution from plants to humans [3]. Members of the *Gimap* gene family are implicated in a variety of basic cellular processes. These include protection against plant pathogens [26] and okadaic acid and gamma radiation [16]. *Gimap* family members have also been found to be important for T cell development [27], B cell activation [28], B cell malignancy [29], and apoptosis [8, 30]. In addition, *Gimap5* deficient mice have been shown to have impaired maturation and survival of CD4 and CD8 positive T cells [13].

In conclusion, positional cloning of the lymphopenia gene in the spontaneously T1D prone BB DP rat revealed a frameshift mutation in the GTPase Immune Associated Protein *Gimap5* [2, 6]. Our coding sequence analysis of the remaining members of the *Gimap* family revealed that *Gimap4* and *Gimap1* each had coding variation between DR.$^{+/+}$ and DR.$^{lyp/lyp}$. However, the amino acid differences do not appear to have functional effects substantiating the *Gimap5* frameshift mutation as the cause of lymphopenia. Quantitative real time PCR analysis showed a reduction in expression of all seven *Gimap* genes in DR.$^{lyp/lyp}$ spleen and mesenteric lymph nodes when compared to DR.$^{+/+}$ while only four, *Gimap1, Gimap4, Gimap5, and Gimap9*, were reduced in thymus. Further understanding of the nature of the *Gimap* family will aid in our goal of characterizing the pathways involved in the development of lymphopenia and T1D.

Acknowledgments

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