



Published in final edited form as:

*J Immunol.* 2013 April 1; 190(7): 3109–3120. doi:10.4049/jimmunol.1203422.

## Genetic interactions among *Idd3*, *Idd5.1*, *Idd5.2* and *Idd5.3* protective loci in the NOD mouse model of type 1 diabetes\*

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### Abstract

In the nonobese diabetic (NOD) mouse model of type 1 diabetes (T1D), insulin-dependent diabetes (*Idd*) loci control the development of insulinitis and diabetes. Independently, protective alleles of *Idd3/II2* or *Idd5* are able to partially protect congenic NOD mice from insulinitis and diabetes, and to partially tolerize islet-specific CD8<sup>+</sup> T cells. However, when the two regions are combined, mice are almost completely protected, strongly suggesting the existence of genetic interactions between the two loci. *Idd5* contains at least three protective sub-regions/causative gene candidates, *Idd5.1/Ctla4*, *Idd5.2/Slc11a1* and *Idd5.3/Acadl*, yet it is unknown which of them interacts with *Idd3/II2*. Through the use of a series of novel congenic strains containing the *Idd3/II2* region and different combinations of *Idd5* sub-region(s), we defined these genetic interactions. The combination of *Idd3/II2* and *Idd5.3/Acadl* was able to provide nearly complete protection from T1D, but all three *Idd5* sub-regions were required to protect from insulinitis and fully restore self-tolerance. By backcrossing a *Slc11a1* KO allele onto the NOD genetic background, we have

\*This work was supported by National Institute of Allergy and Infectious Disease (NIAID) grant AI 070351. X.L. is supported by a graduate student fellowship from The Scripps Research Institute (TSRI) Kellogg School of Science and Technology and by a TSRI Dean's fellowship. E.H.W. was supported by postdoctoral fellowships from the Juvenile Diabetes Research Foundation (JDRF). L.S.W. is supported by the Wellcome Trust (096388) and the Juvenile Diabetes Research Foundation International (9-2011-253). The Cambridge Institute for Medical Research is the recipient of a Wellcome Trust Strategic Award (100140). The content of this article is solely the responsibility of the authors and does not necessarily represent the official views of National Institute of Allergy and Infectious Disease or the National Institutes of Health.

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### Author Contributions

XL designed the study, performed experiments, analyzed data, prepared figures, and wrote the manuscript. EHW designed the study, performed experiments, analyzed data and revised the manuscript. DBR, KMH, LSW and LBP developed congenic mouse strains, performed experiments, analyzed data, prepared figures and revised the manuscript. JC maintained the murine lines and performed experiments. YDD developed congenic mouse strains and revised the manuscript. LSW and LAS conceived and designed the study, analyzed data, and revised the manuscript.

demonstrated that *Slc11a1* is responsible for the diabetes protection resulting from *Idd5.2*. We also used *Slc11a1* KO-SCID and *Idd5.2*-SCID mice to show that both loss-of-function alleles provide protection from insulinitis when expressed on the SCID host alone. These results lend further support to the hypothesis that *Slc11a1* is *Idd5.2*.

## Introduction

T1D is a complex genetic disease caused by genetic interactions amongst numerous genes and influenced by various environmental factors (1–4). In humans, over 50 T1D-associated genes or gene regions have been identified (5–9). A number of genes/gene pathways have been found in common between human T1D and T1D susceptible NOD mice including those encoding HLA/MHC, insulin, IL-2/IL-2R, CTLA-4, PTPN22 and SLC11A1 (3, 4, 10–13). In the human population, the effect size of most T1D-associated genes are small (odds ratio <1.5) and genetic interactions between T1D-associated genes are near impossible to detect (14). However, similar gene variants are found to have large effects on disease frequency in NOD mice, where the background genome is held constant except for the region under investigation (15–18). These regions are named insulin-dependent diabetes (*Idd*) loci, amongst which the *Idd3* and the *Idd5* regions, when combined, provide nearly complete protection from T1D, insulinitis, and the development of autoantibodies (16–19).

Introduction of B6 derived alleles at the *Idd3* region on chromosome 3 onto the NOD parental strain results in significant protection from T1D (20). Using positional cloning, the *Idd3* congenic interval has been narrowed to a 650 kb region that includes the genes encoding IL-2, IL-21, and several other genes (21–25). Yamanouchi and colleagues provided evidence that the IL-2 gene is *Idd3* by correlating disease incidence with the amount of expression of IL-2 (25). Among the six *Idd3* haplotypes tested in this study, no association between *Ii21* production and diabetes protection was observed (25), although two other studies reported that the expression of *Ii21* correlated with *Idd3* haplotype (26, 27). Nonetheless, it is still under debate whether the expression of *Ii2* influences the expression of *Ii21*, which in turn signals APCs that promote Th17 cell development. Both the IL-2 and IL-2 receptor alpha chain genes are associated with human T1D (9, 28) and the *IL2* susceptibility allele is associated with a younger age of disease onset (14). A decrease in IL-2 availability in NOD mice is thought to result in impaired regulatory T cell (Treg) function (25). Interestingly, *Idd3* has also been shown to function outside of the lymphocyte compartment most likely in dendritic cells (DCs) (29, 30).

When B10 derived alleles of the *Idd5* region on chromosome 1 are introduced onto the NOD strain, significant protection from T1D results (16). It is now known that the *Idd5* region consists of at least three *Idd* loci, namely, *Idd5.1*, *Idd5.2*, and *Idd5.3* (16, 18). The *Idd5.1* congenic interval contains two notable candidate genes, *Ctla4* and *Icos*. The *Idd5.2* congenic interval contains *solute carrier family 11 member A1* (*Slc11a1*, previously *Nramp1*) (17). *Idd5.3* contains 11 genes including the candidate gene *Acadl* (18, 31).

Evidence that *Ctla4* is *Idd5.1* includes that human T1D was found to be most strongly associated with a region immediately 3' of the *CTLA4* structural gene, (32, 33). Functional studies demonstrated that the resistant allele of *Ctla4* produces more ligand-independent splice isoform of CTLA-4 (liCTLA-4) than does the disease-associated alleles (32), and that liCTLA-4 mediates negative signaling in T cells (34). liCTLA-4 transgenic NOD mice were found to be protected from T1D to the same extent as *Idd5.1* congenic mice (35). Lastly, haplotype analysis and the development of *Idd5.1* congenic strains that differ at the disease-susceptible *Ctla4* exon 2 single nucleotide polymorphism (SNP) found that increased expression of liCTLA-4 correlated with reduced T1D frequency (35).

*Slc11a1* is the most compelling T1D candidate gene located in the *Idd5.2* region, since a functional missense polymorphism distinguishes the NOD diabetes-susceptible wild-type (WT) allele and the B10 diabetes-resistant loss-of-function mutant allele (17). In humans, *SLC11A1* is associated with T1D and rheumatoid arthritis (11, 36, 37). SLC11A1 is a divalent cation transporter expressed on the membrane of late endosomes and lysosomes in macrophages and DCs, and expression of the WT SLC11A1 confers resistance to intracellular pathogens by promoting phagocytosis and rapid acidification of phago/lysosomes (38–44). By lentiviral transgenesis of congenic NOD mice, *in vivo* knock-down of *Slc11a1* provided protection from T1D as well as susceptibility to *Salmonella* infection, supporting the hypothesis that *Slc11a1* is *Idd5.2* (45). Subsequent studies showed that WT *Slc11a1* expressed in the DCs enhanced antigen processing and presentation (44, 46).

Microarray analysis of activated CD4<sup>+</sup> T cells of NOD mice and *Idd5.3* congenic mice, found *Acadl* to be the most differentially expressed gene, establishing *Acadl* as the strongest gene candidate for *Idd5.3* (31). *Acadl* encodes acyl-coenzyme A dehydrogenase, long chain, an enzyme that catalyzes the first step of fatty-acid  $\beta$ -oxidation (47), but has no known direct immune function. However, considering that activated T cells require enormous expenditures of energy to divide and/or perform their functions and fatty acid metabolism occurs preferentially in naïve and memory but not effector T cells (48–50), it was suggested that *Acadl* may affect the T cell function and survival by altering the efficiency of energy utilization (31).

Previously, studies have found evidence of potent genetic interactions between the *Idd3* and *Idd5* protective alleles (16, 18, 51). Since the *Idd5* protective region consists of three protective sub-regions, in the current study, we set out to identify which *Idd5* sub-regions were required for this interaction. In a prior study, we showed that *Idd3* and *Idd5.1* interactions did not result in increased protection from diabetes (18). We have now developed a series of novel congenic mice isolating the *Idd5.2* and *Idd5.3* regions and recombining the isolated regions back together to verify that an additional *Idd* gene was not evident in between these two regions. These strains were crossed with *Idd3* mice to create various combinations, and the frequency of diabetes, the degree of insulinitis and islet-specific T cell tolerance were investigated. Furthermore, to substantiate the candidacy of *Slc11a1* being *Idd5.2*, we backcrossed the 129-derived *Slc11a1* KO allele onto the NOD genetic background.

## Materials and Methods

### Congenic mouse strains

All experimental procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (IACUC #09-0074). The breeding and genotyping strategies to develop the congenic murine strains with various *Idd* protective alleles have been described (22). For clarity, all the murine strains pertinent to the current study are summarized in Fig. 1, and all the novel markers developed for genotyping are summarized in Supplemental Table I. NOD and NOD-SCID mice were purchased from Taconic. Lines 1094, 1098, 1595, 2193, 3700, 6109, 6359, 6360, 7341 and 10580 were previously described (16–18, 23). Nine novel congenic strains have been developed specifically for this study. Two *Idd5.2* congenic Lines 3388 and 6146 were generated from Line 1595. *Idd5.3* congenic Line 7345 was generated from Line 6360. *Idd5.2+5.3* congenic Line 7341, in which the isolated *Idd5.2* and *Idd5.3* congenic intervals were combined, was generated from crossing Line 6146 and Line 6360. *Idd3+5.2* congenic Line 6096 was generated by crossing Line 1098 with Line 3388. Two *Idd3+5.3* congenic Lines 6259 and 9246 were generated by crossing Line 1098 with Lines 3700 that has the *Idd5* segment shown for Line 6259 in Fig. 1 (18) and 7345. Two *Idd3+5.2+5.3* congenic Lines 7380 and

9245 were generated by crossing Line 1098 with Lines 1595 and 7341. Lines 6109, 2193, 7341, 6146, 7345, 1094, 2574 and 1098 have been cryopreserved at The Jackson Laboratory ([www.jaxmice.jax.org](http://www.jaxmice.jax.org)) and are available as lines 012393, 012391, 012396, 012394, 012395, 004344, 012392 and 007934 respectively.

To further investigate the strongest candidate gene for *Idd5.2* allele, namely, *Slc11a1*, two novel NOD.129 murine strains were developed (Fig. 1). Line 8848 NOD.129 *Slc11a1* WT (*Slc11a1* WT) was developed by backcrossing the 129S6 strain (Taconic) 9 times with the NOD/MrkTac (Taconic) strain, selectively breeding progeny with proximal and distal recombination events close to the *Slc11a1* gene: the 129-derived region of interest is defined by the proximal in and out markers rs30495565 and rs32083833, respectively, and the distal in and out markers rs30150991 and rs32807660, respectively. Line 10071 NOD.129 *Slc11a1* knockout (*Slc11a1* KO) was developed in a similar manner by backcrossing *Slc11a1*-null 129S4 mice (46, 52) 11 times with the NOD strain; the 129-derived region of interest is defined by the proximal in and out markers rs32912547 and rs30495565, respectively, and the distal in and out markers D1mit46 and rs30150991, respectively. A 1449 SNP marker panel across 19 autosomes and the X chromosome, averaging a genetic interval of 5 Mbps (1449 marker panel, Taconic) was used to identify and remove by selective breeding any remaining 129-derived regions other than the desired congenic interval.

*Idd5*-SCID congenic strains have been described previously (30). *Idd5.2*-SCID mice were generated by inter-crossing Line 6146 and NOD-SCID mice and selecting mice homozygous for both alleles. *Slc11a1* KO-SCID was generated by inter-crossing *Slc11a1* KO and NOD-SCID mice and selecting progeny homozygous for both alleles.

### Variant identification in *Idd5.2*

The NOD and 129 genomes have been sequenced using next generation sequencing technology (<http://www.sanger.ac.uk/resources/mouse/genomes/>). Single nucleotide polymorphisms (SNPs) from the B6, NOD and 129 *Idd5.2* region were entered into T1DBase (53, 54) and displayed graphically using Gbrowse (55).

### Reconstitution of SCID mice

As previously described (30), female SCID mice were reconstituted with total spleen and lymph node cells prepared from female donor mice 3 to 5 weeks of age. DCs were depleted with Pan-DC microbeads (Miltenyi Biotec) according to the manufacturer's instructions before transfer of  $2-3 \times 10^7$  cells intravenously. Mice were rested for 10 weeks before harvesting pancreata.

### Assessment of diabetes and insulinitis

Female mice were monitored on a bi-weekly basis, and mice having elevated urinary glucose  $>500$  mg/dl (detected using Diastix (Myles, Elkhart, IN)) for two consecutive daily tests were classified as diabetic and euthanized thereafter. During the diabetes frequency study, a few non-diabetic mice became sick due to infection or a tumor, or were found dead with unknown cause and these were censored from the analysis. The diabetes frequency studies represented in Figures 2A and 2D were performed at Merck Research Laboratories whilst the studies in Figures 2B, 2C, 2E5B and supplemental Fig. 1 were conducted at Taconic. The diabetes frequency of the control NOD strain was notably reduced in the study reported in Fig. 5B due to unknown environmental influences since the genetic integrity of the NOD strain used at Taconic for the study depicted in Fig. 5B was confirmed.

Pancreata were fixed in 10% neutral buffered formalin, processed for paraffin embedding, stained with hematoxylin and eosin, and cut to obtain two non-contiguous tissue sections.

Blinded tissue sections were evaluated for the presence of mononuclear cell infiltrates in the pancreatic islets. At least 20 islets were scored as either 0: no infiltration, 1: peri-insulinitis, 2: mild-invasive insulinitis, or 3: severe invasive insulinitis. The average score of each pancreas was calculated and used for statistical analysis.

## Virus

Recombinant vaccinia virus (Vac-K<sup>d</sup>IGRP) expressing the H-2K<sup>d</sup> restricted epitope VYLKTNVFL, amino acid residues 206–214 of murine Islet-specific glucose-6-phosphatase catalytic subunit related protein (IGRP) was previously described (56). Mice were infected intraperitoneally with  $1 \times 10^7$  p.f.u. of virus and CD8<sup>+</sup> T cell responses measured in the spleen 7 days later by tetramer staining.

## Flow cytometry

CD8<sup>+</sup> T cells were stained with H-2K<sup>d</sup>-IGRP<sub>206-214</sub>-PE tetramers (NIAID MHC Tetramer core facility) for 15 minutes RT followed by staining with anti-CD8-FITC at 4°C for 15 minutes. All mAbs were obtained from either eBioscience (San Diego, CA), BioLegend (San Diego, CA) or BD Pharmingen (San Diego, CA). Cells were acquired with either a FACS Calibur or LSRII (Becton Dickinson, Mountain View, CA.) and analyzed with FlowJo software (Tree Star, Inc., Ashland, OR).

## Statistical analysis

Kaplan Meier survival analysis of diabetes was performed for each group of mice and T1D frequencies were compared using the log-rank test (Graphpad Prism software). Differences in the degrees of insulinitis were compared between groups via the Mann-Whitney test (Graphpad Prism software). Differences in the proportion or number of IGRP tetramer binding cells were compared between groups via the unpaired student t-test with Welch's correction (Graphpad Prism software).

## Results

### Recombining isolated *Idd5.2* and *Idd5.3* congenic intervals recapitulates the T1D protection observed with contiguous protective alleles

The previous observation of approximately 2% diabetes frequency amongst *Idd3/5* females strongly suggests that potent interlocus genetic interactions between *Idd3* and *Idd5* exist (16, 18, 51). In our previous study (18), we combined the isolated protective *Idd5.1* and *Idd3* regions in a double congenic strain, and showed that the *Idd3+5.1* strain and *Idd3* strain were equally protected from T1D, and that both strains were significantly less protected from T1D than *Idd3/5* strain, suggesting that either *Idd5.2*, or *Idd5.3*, or both, are essential for the interlocus genetic interactions with *Idd3*. However, concurrent with the development of such double and triple congenic strains combining *Idd5.2*, or *Idd5.3*, or both, with *Idd3* (18), we wanted to include a comparison of the diabetes protection provided by the *Idd5.2* and *Idd5.3* regions together on a single congenic segment (Line 1595, Fig. 1) to that of the isolated congenic regions recombined back together (Line 7341) in the presence or absence of protective *Idd3* alleles. This strategy would enable us to determine if another *Idd* subregion is present between the *Idd5.2* and *Idd5.3* regions as did a similar experiment of recombining the isolated *Idd5.1* and *Idd5.2* regions facilitated the discovery of the *Idd5.3* region (18). In pursuing this strategy, we developed novel strains having the isolated *Idd5.2* and *Idd5.3* congenic intervals. These novel strains enabled us to confirm the T1D protection using independently-derived congenic segments and to reduce the overall size of the introgressed congenic interval as compared to the analogous strains previously published.

Two novel *Idd5.2* congenic strains were developed, Lines 3388 and 6146 (Fig. 1). We observed that both strains were indistinguishable from each other in their respective T1D frequency and significantly more protected than control NOD mice (Fig. 2A). This confirms our previous findings (18, 45) that the presence of a B10-derived congenic region encompassing the 1.5 Mb *Idd5.2* interval including the candidate gene *Slc11a1* (17) was sufficient to confer highly significant, albeit moderate, protection from T1D, compared with NOD strain (Fig. 2A).

We also developed a novel *Idd5.3* congenic Line 7345, and observed that its T1D frequency was indistinguishable from that of Line 6360 (Fig. 2B), an *Idd5.3* congenic strain that we developed in a previous study (18). This demonstrated that the presence of the B10-derived resistance allele at *Idd5.3* locus alone was sufficient to confer highly significant, albeit moderate, protection from T1D, compared with NOD strain (Fig. 2B). The protective effect of Line 7345 corroborates previous work mapping *Idd5.3* to a 3.5 Mb interval including the candidate gene *Acadl* (18, 31).

We developed a novel *Idd5.2+5.3* congenic Line 7341 in which the isolated *Idd5.2* (from Line 6146) and *Idd5.3* (from Line 6360) congenic intervals were combined by selective breeding of an inter-subregion recombinant (Fig. 1). We observed that the T1D frequency of Line 7341 was indistinguishable from that of Line 1595 (Fig. 2C), an *Idd5.2+5.3* congenic strain that we developed in a previous study where the *Idd5.2* and *Idd5.3* regions are both in the same congenic segment (18), indicating that it is unlikely there are additional *Idd* genes located between the *Idd5.2* and *Idd5.3*. The two *Idd5.2+5.3* congenic strains were compared to Line 6359, which contains the proximal end of Line 1595, and was previously shown to have a diabetes incidence similar to NOD mice (18). The high incidence of Line 6359 found here (Fig 2C) replicates the finding that Line 6359 does not contain a protective gene and *Idd5.3* is located in the distal half of the congenic segments present in Lines 6360 and 7345.

### Interlocus genetic interaction between *Idd3* and *Idd5* sub-regions in the protection from T1D

We now combined the *Idd5.2*, *Idd5.3* and *Idd5.2+5.3* congenic intervals with *Idd3* in order to test which region(s) are required for the interlocus genetic interaction and prevention of diabetes. The *Idd5.2* congenic Line 3388 was crossed with *Idd3* mice to generate a novel *Idd3+5.2* congenic Line 6096 (Fig. 1). Line 3700, an *Idd5.3* congenic strain that we developed in a previous study (18) that has the *Idd5* segment shown for Line 6259 in Fig. 1, was crossed with *Idd3* mice to generate a novel *Idd3+5.3* congenic Line 6259 (Fig. 1). The *Idd5.3* congenic Line 7345 was crossed with *Idd3* mice to generate another novel *Idd3+5.3* congenic Line 9246 (Fig. 1). Both *Idd5.2+5.3* congenic Lines 1595 and 7341 were crossed with *Idd3* mice to generate two novel *Idd3+5.2+5.3* congenic Lines 7380 and 9245 (Fig. 1).

The diabetes frequency of the *Idd3+5.2* congenic Line 6096 was indistinguishable from that of its contemporaneous control *Idd3* mice (Fig. 2D), suggesting that *Idd5.2* is not sufficient for the interlocus genetic interactions when diabetes phenotype was assessed. The diabetes frequency of the *Idd3+5.3* congenic Line 6259 was indistinguishable from that of its contemporaneous control *Idd3* mice, although we observed a trend of *Idd3+5.3* strain having less incidence of disease than *Idd3* strain (Fig. 2D). The diabetes frequency of the more recently developed *Idd3+5.3* congenic Line 9246 was significantly lower than that of its contemporaneous control *Idd3* mice, and was indistinguishable from that of its contemporaneous control *Idd3/5* mice (Fig. 2E). Considering the stochastic nature of the onset and development of T1D, we assessed both studies together in a combined analysis (Supplemental Fig. 1). The combined *Idd3+5.3* mice were significantly more protected from T1D than the control *Idd3* mice (Supplemental Fig. 1), suggesting that that *Idd5.3* is essential for the interlocus genetic interactions when diabetes phenotype was assessed.

The disease frequencies of *Idd3+5.2+5.3* congenic Lines 7380 and 9245, as well as their contemporaneous control *Idd3/5* mice, were next assessed and found to be indistinguishable from each other (Fig. 2E). All three strains were significantly more protected from T1D than the contemporaneous control *Idd3* mice (Fig. 2E), providing strong support to our above mentioned hypothesis that *Idd5.3* is essential for the interlocus genetic interactions when diabetes phenotype was assessed.

### Genetic interactions between *Idd3* and *Idd5* sub-regions that lend protection from insulinitis

Our previous observation that *Idd3+5.1+5.3* females were protected from disease to a degree indistinguishable from that of *Idd3/5* females (18) appears to suggest that *Idd5.2* is not essential for the interlocus genetic interactions with *Idd3*. However, when the occurrence and severity of insulinitis of the two strains were compared, it was discovered that *Idd3+5.1+5.3* mice had significantly more insulinitis than *Idd3/5* mice (18), indicating that *Idd5.2* is required for the interlocus genetic interactions with *Idd3* to reduce insulinitis. Hence, in regard to the NOD genetic background, although both diabetes and insulinitis have a certain degree of stochastic nature, the insulinitis readout tends to provide a larger window and more power than the diabetes readout, in distinguishing a relatively smaller yet clearly existing difference between two different strains.

In order to further investigate the genetic interactions between *Idd3* and *Idd5* sub-regions that provide protection from insulinitis, five strains: *Idd3* mice, *Idd3+5.3* congenic Line 9246, *Idd3+5.2+5.3* congenic Lines 7380 and 9245, and *Idd3/5* mice, were examined for the occurrence and severity of insulinitis (Fig. 3). The latter four strains were specifically selected because among all the newly developed strains, they were the only four that were significantly more protected from diabetes than their contemporaneous *Idd3* controls (Fig. 2E). The occurrence and degrees of insulinitis of both *Idd3+5.2+5.3* congenic Lines 7380 and 9245 were indistinguishable from each other (Supplemental Fig. 2); therefore, we combined these two strains in subsequent analyses (Fig. 3). Reminiscent of our previous study (18), a hierarchy in the protection from insulinitis was discovered: first, *Idd3/5* strain was more protected than *Idd3+5.2+5.3* strains (Fig. 3), suggesting that *Idd5.1* contributes to reduced insulinitis in the presence of *Idd3*, *Idd5.2* and *Idd5.3* protective alleles; second, *Idd3+5.2+5.3* mice were more protected than the *Idd3+5.3* and *Idd3* mice (Fig. 3), suggesting that *Idd5.2* contributes to reduced insulinitis in the presence of *Idd3* and *Idd5.3* protective alleles in the NOD genome; third, *Idd3+5.3* mice and *Idd3* mice had equivalent levels of insulinitis (Fig. 3), suggesting that although *Idd5.3* contributes to reduced diabetes in the presence of *Idd3* (Fig. 2E and Supplemental Fig. 1), it does not contribute to insulinitis reduction. It is important to note that *Idd3+5.3* mice, the two lines of *Idd3+5.2+5.3* mice and *Idd3/5* mice had indistinguishable T1D frequency (Fig. 2E), yet the first three strains all had significantly more occurrence and greater severity of insulinitis than *Idd3/5* strain (Fig. 3). Taking into account both diabetes incidence and insulinitis, we conclude that all three *Idd5* sub-loci on chromosome 1 are required to interact with *Idd3* locus on chromosome 3 to provide protection from diabetes and insulinitis to the extent observed in *Idd3/5* mice.

### *Idd5.2* alone is able to partially restore self-tolerance to endogenous islet antigen IGRP

IGRP is an endogenous pancreatic islet antigen. The number of IGRP specific CD8<sup>+</sup> T cells is an indication of auto-reactivity and loss of self-tolerance, and is predictive of T1D progression in individual NOD mouse (57). Compared with the NOD strain, *Idd3/5* mice exhibited noticeably restored CD8<sup>+</sup> T cell tolerance (29, 58, 59). Previously, we found that both the *Idd3* and *Idd5* regions independently contributed to the partially restored CD8<sup>+</sup> T cell tolerance, and that their effects were additive (30).

The development of isolated *Idd5* sub-region strains allowed us to investigate which of the three *Idd5* sub-regions contribute to restored CD8<sup>+</sup> T cell tolerance observed in *Idd5* congenic strain. Hence, we applied the same method to characterize the CD8<sup>+</sup> T cell tolerance to IGRP in these strains (Fig. 4A). Consistent with our previous report (30), *Idd5* mice exhibited a significantly reduced percentage of IGRP-specific CD8<sup>+</sup> T cells (Fig. 4A), suggesting that the entire *Idd5* genetic segment contributes to the restoration of CD8<sup>+</sup> T cell tolerance. Among the three *Idd5* sub-regions, only the *Idd5.2* region significantly reduced the percentage of IGRP-specific CD8<sup>+</sup> T cells, to the same degree as that of the *Idd5* strain (Fig. 4A). The effect of the *Idd5.2* region was also observed in the *Idd5.2+5.3* strain, which showed significantly reduced percentage of IGRP-specific CD8<sup>+</sup> T cells, to the same degree as that of the *Idd5* strain (Fig. 4A).

#### **When combined with *Idd3*, more than one *Idd5* sub-regions is needed to restore self-tolerance to the same degree observed in *Idd3/5* mice**

The development of *Idd3+5.1*, *Idd3+5.2*, *Idd3+5.3* and *Idd3+5.2+5.3* strains presented an opportunity to investigate the potential interactions between each of three *Idd5* sub-regions and *Idd3* for restoration of CD8<sup>+</sup> T cell tolerance. Consistent with our previous report (30), *Idd3/5* mice exhibited a significantly reduced percentage of IGRP-specific CD8<sup>+</sup> T cells than that of *Idd3* mice (Fig. 4B), suggesting that at least one gene within *Idd5* contributes to the genetic interactions with *Idd3*. Since both *Idd3+5.3* congenic Lines 6259 and 9246 were indistinguishable from each other (Supplemental Fig. 3), we combined them in the subsequent analyses. Both *Idd3+5.2+5.3* congenic Lines 7380 and 9245 were also indistinguishable from each other (Supplemental Fig. 3), and were combined in the subsequent analyses. None of the three *Idd5* sub-regions, when individually combined with *Idd3*, was able to significantly reduce the percentage of IGRP-specific CD8<sup>+</sup> T cells (Fig. 4B). However, the combination of *Idd5.2* and *Idd5.3* sub-regions was able to interact with *Idd3* to provide additional restoration of CD8<sup>+</sup> T cell tolerance, to a degree that was indistinguishable from that of *Idd3/5* mice (Fig. 4B). These results suggest that more than one *Idd5* sub-region is required to interact with *Idd3* to further restore CD8<sup>+</sup> T cell tolerance.

#### **NOD.129 *Slc11a1* KO strain is protected from T1D and shows partially restored self-tolerance to endogenous islet antigen IGRP**

As the protective B10 allele at *Slc11a1* is a loss of function mutation (17), we reasoned that if *Slc11a1* was the causative gene in the *Idd5.2* locus, *Slc11a1* KO NOD mice should be protected from T1D. Hence, we crossed a *Slc11a1*-null allele from the 129S4 genetic background (46, 52) onto the NOD genetic background and generated a novel *Slc11a1* KO NOD strain. As a control, we crossed the *Slc11a1*-WT allele from 129S6 mice onto the NOD genetic background. The control strain expresses the functional *Slc11a1* allele in the context of the 129 genome at the *Idd5.2* region matching closely to the 129-derived congenic interval present in the NOD.129 *Slc11a1*-null strain (Fig. 1 and Fig. 5A). The two congenic 129 intervals are not identical in size as it is nearly impossible by chance to achieve identical recombination sites between two strains. However, the congenic regions in both strains encompass the previously defined *Idd5.2* region shown as a horizontal arrow in Fig. 5A. The gene content and SNP density of the congenic intervals are also shown in Fig. 5A.

After the development of these two strains, we first tested the diabetes incidence, in comparison to contemporaneous control NOD (Fig. 5B). Diabetes frequency of *Slc11a1* WT was indistinguishable from NOD (Fig. 5B), even though NOD and 129 have different haplotypes across this region (Fig. 5A). This observation strongly suggests that the DNA segment derived from the 129 genetic background carrying *Slc11a1* WT alleles does not



provide significant protection from diabetes, and SNPs in other 129-derived genes in the congenic region also do not influence disease susceptibility. In contrast, diabetes frequency of *Slc11a1* KO was significantly lower than that of *Slc11a1* WT and NOD controls (Fig. 5B). Since the *Slc11a1* KO 129 congenic segment is contained entirely within the WT 129 congenic segment, the T1D frequencies observed indicate that the *Slc11a1* WT alleles in the context of NOD genome are T1D susceptibility alleles and *Slc11a1* KO alleles confer protection from diabetes. It is also notable that where the two congenic strains differ, the NOD and 129 strains either share the same sequence (proximal boundary region) or no coding genes are present (distal boundary region); this is consistent with the hypothesis that the *Slc11a1* disruption is the genetic event causing reduced diabetes susceptibility rather than a complex interaction amongst two or more 129-derived genes that are variably present in the two NOD.129 congenic strains and that *Idd5.2* is *Slc11a1*.

As the *Idd5.2* strain provides partial restoration of CD8<sup>+</sup> T cell tolerance to IGRP, we reasoned that the *Slc11a1* KO allele on the NOD genetic background should also mediate this effect. However, the examination of young female mice 10–12 weeks of age revealed no difference between the percentages of IGRP specific CD8<sup>+</sup> T cells of the *Slc11a1* WT and KO strains (Fig. 5C). This was a surprising result, considering that the diabetes frequencies of the two strains clearly differ from each other (Fig. 5B). Therefore, we decided to assess older female mice, 14–17 weeks of age, as unpublished data (E.H.W. personal observations) strongly indicates that IGRP specific CD8<sup>+</sup> T cells accumulate in various NOD congenic mice as they age. We found that comparing the mice at 14–17 weeks of age, *Slc11a1* KO has significantly reduced percentage of IGRP specific CD8<sup>+</sup> T cells than *Slc11a1* WT (Fig. 5C), suggesting the *Slc11a1* KO alleles contribute to a partial restoration of CD8<sup>+</sup> T cell tolerance.

### ***Slc11a1* loss of function alleles expressed in the SCID hosts provides insulinitis protection**

SLC11A1 is a divalent cation transporter that is exclusively expressed in macrophages and DCs (29, 46). Therefore, we tested if the expression of either the B10-derived *Idd5.2* alleles encompassing the gene encoding the loss of function mutant SLC11A1 protein, or the *Slc11a1* KO alleles in non-lymphocytes would provide protection against insulinitis, by reconstituting NOD-SCID, *Idd5*-SCID, *Idd5.2*-SCID, and *Slc11a1* KO-SCID with DC-depleted lymphocytes purified from young *Idd3* congenic donor mice. The rationale of using *Idd3* donor lymphocytes was that the incomplete protection from insulinitis provided by the genes in the donor cells allows the detection of insulinitis protection conferred by the genes in the host (30). As shown in Fig. 6, the presence of either *Idd5* or *Idd5.2* in non-lymphocytes conferred significant protection from insulinitis, and the degree of insulinitis protection provided by *Idd5* or *Idd5.2* was indistinguishable, suggesting that the insulinitis protection conferred by *Idd5* should be attributed to *Idd5.2*, and that *Idd5.1* and *Idd5.3* alleles were not required. Notably, *Slc11a1* KO-SCID mice were also strongly protected from insulinitis, compared to NOD-SCID mice (Fig. 6), providing further evidence that *Slc11a1* is the causative gene in the *Idd5.2* region.

## **Discussion**

The *Idd3/5* mouse on the NOD genetic background contains the B6-derived *Idd3* region on chromosome 3 and B10-derived *Idd5* region on chromosome 1, and it was nearly completely protected from diabetes and insulinitis (16, 18). Cordell and colleagues modeled the interlocus genetic interactions of protective alleles at *Idd3* and *Idd5*, and concluded that synergistic gene-gene interactions between two loci were evident (51). The combination of *Idd3* and *Idd5* protective alleles has also been shown to potently resist autoimmunity in other experimental conditions. For instance, an AIRE knockout crossed onto the B6 genetic background resulted in minimal autoimmunity, on the NOD genetic background, it resulted

in very severe multi-organ autoimmunity (60); however, the combination of *Idd3* and *Idd5* protective alleles was found to abrogate the severe autoimmunity caused by the AIRE knockout on the NOD genetic background (60).

As reviewed in Ridgway *et al.*, a major obstacle in identifying the disease-causing gene variants and their molecular mechanisms acting in the immune system is that many of those candidate genes function in more than one cell types, some of which could have opposing effects during the course of the development of self-tolerance or autoimmunity (3). Yamanouchi *et al.* provided strong evidence that the reduced IL-2 production by the NOD *Idd3/II2* allele correlates with reduced suppressor function of Tregs (25), and this correlation was confirmed in another study (61). However, IL-2 may well play a pivotal role in not only Tregs, but also DCs (30). It was shown that DCs stimulated with microbial antigens are able to produce IL-2 and prime T cells (29, 62). An unorthodox mechanism by which CD25 molecules expressed on the surface of mature DCs could present IL-2 produced by the DCs to prime the T cells and to enhance the T cell activation was recently described (63).

Functional studies demonstrated that the protective allele of *Idd5.1* produces more ligand-independent splice isoform of CTLA-4 (liCTLA-4) than does the disease-associated alleles (32). The liCTLA-4 molecules mediate negative signaling in T cells by dephosphorylating the TCR zeta chain (34) and increased expression of liCTLA-4 correlated with reduced T1D frequency (35). Since *Ctla4* is a critical gene expressed exclusively in lymphocytes (17), *Idd3* and *Idd5.1* could act together in Tregs to enhance their suppressor function.

The most compelling candidate gene within *Idd5.2*, *Slc11a1*, encodes a cation transporter expressed on the phago-lysosomal membrane of DCs and macrophages (38–44). There is a known functional missense polymorphism (Gly<sup>169</sup>) > (Asp<sup>169</sup>) that distinguishes the disease-associated WT allele and the disease-protective loss-of-function mutant allele (17). Functional studies demonstrated that, compared with the loss-of-function mutant allele, WT *Slc11a1* expressed in the DCs enhanced antigen processing and presentation (44, 46), induced more Th1 cytokine production by T cells (46), increased the proliferation of diabetogenic T cells (46), and that *in vivo* knock-down of WT *Slc11a1* provided congenic mice on the NOD genetic background with significant protection from T1D (45). Considering the above discussed effects of IL-2 production by DCs, it is possible that *Idd3* and *Idd5.2* could act together in DCs to enhance self-tolerance.

The strongest candidate gene within *Idd5.3* is *Acadl*, encoding acyl-coenzyme A dehydrogenase (31), an enzyme that catalyzes the first step of fatty-acid  $\beta$ -oxidation (47). Mice carrying *Acadl* KO alleles had severe defects in maintaining body temperature (64–66). It was shown that activated T cells require enormous energy to divide and/or carry out their functions, and that fatty acid metabolism occurs preferentially in naïve and memory but not effector T cells (48–50). It was proposed that *Idd5.3* may affect the T cell function and survival by altering fatty acid metabolism (31). Therefore, it is possible that *Idd3*, *Idd5.1*, and *Idd5.3* could interact in Tregs; it is also possible that *Idd3*, *Idd5.2*, and *Idd5.3* could interact in DCs, considering that ACADL is an enzyme ubiquitously expressed in all cell types.

Of the new bi-congenic strains, only *Idd3+5.3* strains were significantly more protected from T1D than the *Idd3* strain, suggesting that that *Idd5.3* is essential for the interlocus genetic interactions with *Idd3* when diabetes phenotype was assessed. Supporting this interpretation, the *Idd3+5.1+5.3* strain (18) and the two *Idd3+5.2+5.3* strains that we developed in this study were all significantly more protected from T1D than the *Idd3* mice. It was surprising that when present alone, among the three *Idd5* sub-regions, *Idd5.3* is the

least protective (18) (Figs. 2A and 2B), but when combined with *Idd3*, *Idd5.3* provides most additional protection from the disease (18) (Figs. 2D and 2E).

Although *Idd3+5.1+5.3* females appeared to be protected from T1D to a degree indistinguishable from that of *Idd3/5* females, they were found to have significantly more insulinitis than *Idd3/5* females (18). Therefore, we concluded that the insulinitis readout tends to be more sensitive in distinguishing a small difference between two different strains, even when they appeared to be equally protected from T1D. Indeed, we found that each of the three *Idd5* sub-regions when combined with the *Idd3* strain provided an additional degree of protection from insulinitis development, highlighting the requirement of the presence of multiple protective alleles for synergistic genetic interactions. Hence, by assessing both diabetes and insulinitis, we concluded that all three *Idd5* sub-regions were required to interact with *Idd3* to provide the nearly complete protection from diabetes and insulinitis as observed in the *Idd3/5* strain.

We have found that the expression of *Idd3* and *Idd5* protective alleles in not only DCs, but also CD4<sup>+</sup> T cells, contributes to the self-reactive CD8<sup>+</sup> T cell deletional tolerance (29). In order to dissect the cellular basis by which one or more *Idd* protective alleles contribute to self tolerance, we examined the extent of restoration of CD8<sup>+</sup> T cell tolerance in the various strains of *Idd3+Idd5*-sub-region congenic mice. We found that only the *Idd3+5.2+5.3* strains and the *Idd3/5* strain had significantly reduced IGRP-specific CD8<sup>+</sup> T cells as compared with the *Idd3* strain, whereas none of the *Idd3+5.1*, *Idd3+5.2*, and *Idd3+5.3* strains had significantly less IGRP-specific CD8<sup>+</sup> T cells than the *Idd3* strain. These observations are similar to the observations that we made when we examined the insulinitis of those strains, highlighting the requirement and necessity of having more than one *Idd5* sub-region interacting with the *Idd3* region to provide significant additional synergistic effects in both situations. Interestingly, the isolated *Idd5.2* strain provided equivalent protection from loss of IGRP-specific CD8<sup>+</sup> T cell tolerance to the full *Idd5* strain. As *Idd5.2/Slc11a1* functions in antigen presenting cells, this is likely due to cognate interactions between the CD8<sup>+</sup> T cells and the antigen presenting DCs.

Although the development of congenic strains that are protected from T1D due to the presence of a genetic interval derived from a T1D-resistant strain is one of the most important tools in defining genes underlying T1D in the NOD mouse model, a limitation is that the genetic segment containing the allele of interest can not be reduced to a length less than 0.5–3 Mb, depending on the recombination frequency of the region under investigation (3). Therefore, in a gene-dense region such as the *Idd5.2* locus that contains at least 45 genes (17), reducing the congenic interval by positional cloning to include only one gene is technically impossible. In order to test the hypothesis that *Slc11a1* is *Idd5.2*, a lentiviral transgenesis and *in vivo* RNA interference strategy was taken to generate an NOD strain in which *Slc11a1* was knocked down by shRNA (45). It was demonstrated that *in vivo* knock-down of *Slc11a1* significantly reduced the T1D incidence (45). However, with the RNA interference experimental approach, it is nearly always impossible to completely eliminate a concern of the possibility of off-target effects (67, 68).

In order to directly test the consequences of lacking *Slc11a1* in the NOD genome, in this study we backcrossed the 129-derived *Slc11a1* KO allele onto the NOD genetic background, and demonstrated significant protection from T1D due to the lack of *Slc11a1*. However, in a complex genetic disease such as T1D, not only disease-promoting genes, but also environmental factors, influence the onset and development of the disease (1, 2). In the case of our T1D frequency study of *Slc11a1* WT, *Slc11a1* KO and the control NOD strains (Fig. 5B), the disease incidence was particularly low compared to our previous studies of other *Idd* congenic strains (18) (Figs. 2A, 2B and 2C). Noticing that even the NOD females of the

control group had a much lower T1D frequency than the same NOD strain in our other T1D frequency studies carried out previously (18) (Figs. 2A, 2B and 2C), we propose that the most likely explanation of the low T1D incidence is the variation of environmental factors.

To further test the hypothesis that *Slc11a1* is *Idd5.2*, we examined the percentage of self-reactive IGRP specific CD8<sup>+</sup> T cells in the *Slc11a1* WT and *Slc11a1* KO strains, and found in young female mice (10–12 weeks of age) there was only a small statistically insignificant difference (Fig. 5C). Since our unpublished data (E.H.W. personal observations) suggest that as the mice age, IGRP specific CD8<sup>+</sup> T cells accumulate in various strains of congenic mice on the NOD background, we chose to compare *Slc11a1* WT and *Slc11a1* KO females at 14–17 weeks of age, and found that *Slc11a1* KO has significantly less self-reactive CD8<sup>+</sup> T cells than *Slc11a1* WT (Fig. 5C), suggesting that *Slc11a1* KO allele functions similarly to *Idd5.2* disease-resistant allele encoding the *SLC11A1* loss-of-function mutant by contributing to a partial restoration of CD8<sup>+</sup> T cell tolerance. As illustrated in Figure 5A, the *Idd5.2* locus is a gene-dense region encoding at least 45 genes (17), and a large number of SNPs exist in that genetic interval between NOD allele and 129 allele, between NOD allele and B6 allele, between B6 allele and 129 allele. It is plausible, even probable, that one or more SNPs, in addition to the disrupted *Slc11a1* gene contribute to the difference in the CD8<sup>+</sup> T cell tolerance as measured by the percentage of self-reactive IGRP specific T cells between the *Slc11a1* KO and *Idd5.2* strains (Figs. 4A and 5C).

Since SLC11A1 is expressed in macrophages and DCs (38–44), we tested whether the lack of functional SLC11A1 expressed in the host would be associated with increased protection from the disease. Although we were unable to successfully discern if the lack of SLC11A1 expression in the host compartment provided protection from diabetes, we found that the 129-derived *Slc11a1* KO allele, the *Idd5.2/Slc11a1* mutant allele, as well as the *Idd5* allele (which contains the *Idd5.2* region) in the host provided significant protection from insulinitis (Fig. 6), a result that strongly supports our hypothesis that *Slc11a1* is *Idd5.2*.

Our study provides new insights into the synergistic genetic interactions among *Idd3/II2*, *Idd5.1/Ctla4*, *Idd5.2/Slc11a1*, *Idd5.3/Acadl*, the first three of which are all associated with human T1D (10–13, 32, 33), emphasizing the common pathogenesis for human and mouse T1D and highlighting the relevance of the NOD and its derived congenic strains for modeling human variants. This study also supports the hypothesis that *Slc11a1* is *Idd5.2*, a finding that highlights SLC11A1 as a molecular target for immune-based therapeutic interventions.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

The authors thank all members of the Sherman laboratory for helpful discussion; Kristi Marquardt for technical assistance; Kathleen Cairns and Michelle Gassert for administrative assistance. We also thank the TSRI histology core facility who prepared histology sections and the TSRI Flow Cytometry core facility for sorting and help with FACS analysis. This is manuscript #22049 from The Scripps Research Institute.

## Abbreviations used in this paper

<i>Acadl</i>	acyl CoA dehydrogenase long chain
DC	dendritic cell

<b>Idd</b>	insulin-dependent diabetes
<b>IGRP</b>	islet-specific glucose-6-phosphatase catalytic subunit related protein
<b>Slc11a1</b>	solute carrier family 11 member a1
<b>T1D</b>	type 1 diabetes
<b>Treg</b>	T regulatory cell.

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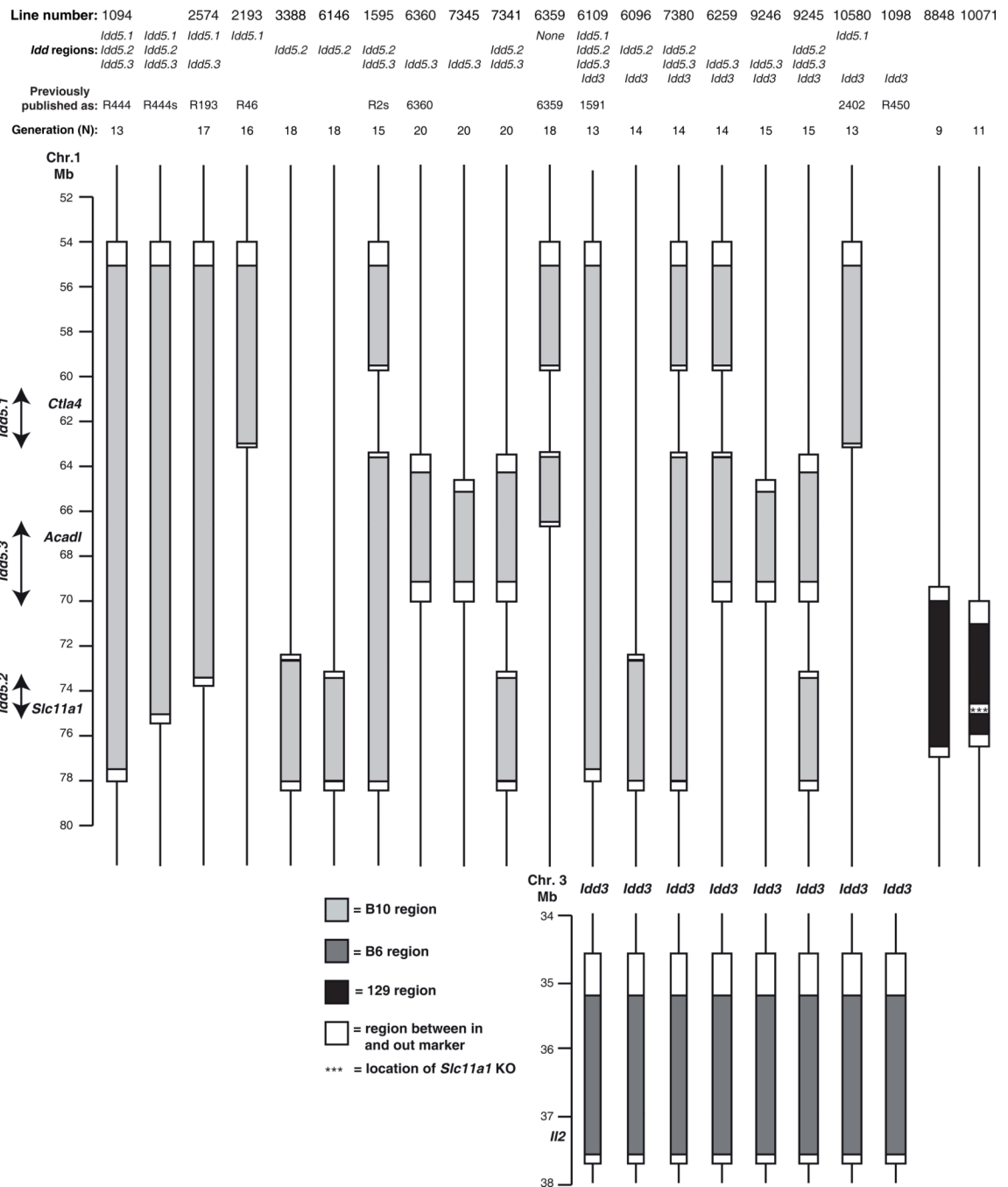
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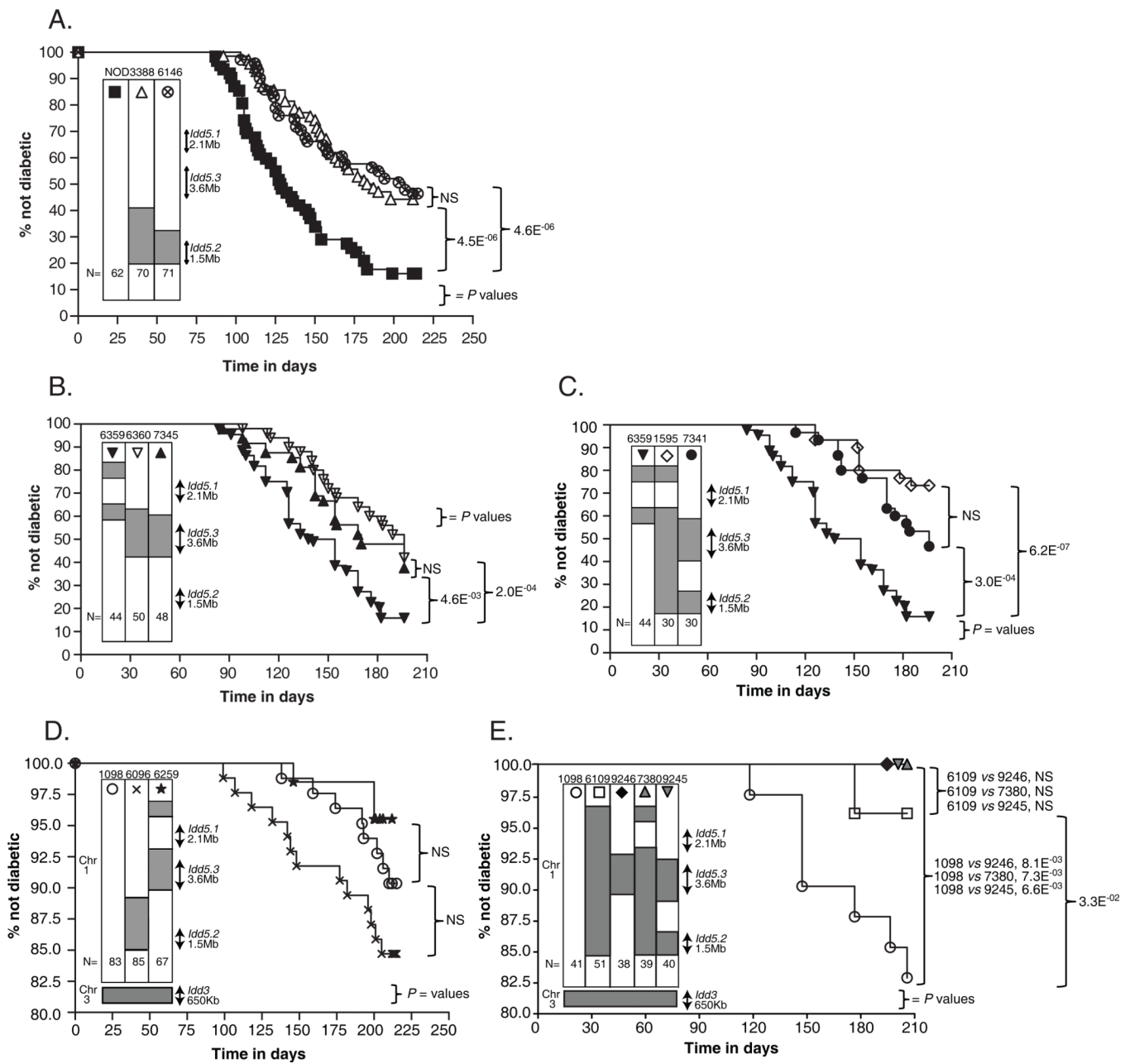
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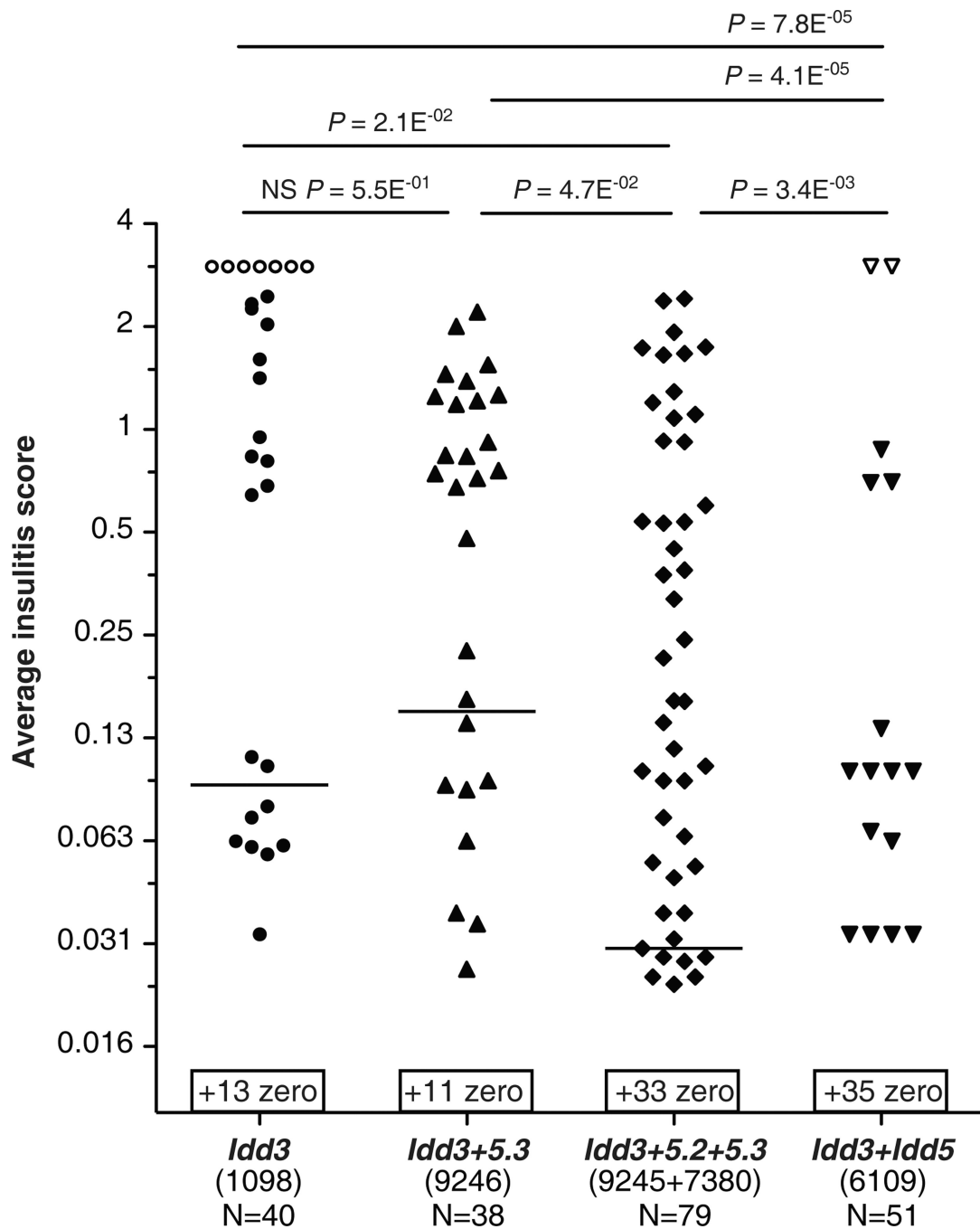


**Figure 1.** Genetic intervals of the strains pertinent to this study. Line numbers, or strain names, as well as the *Idd* regions that the strains carry respectively, were labeled on top of the figure. Generation (N) indicates the number of generations of backcrossed to parental NOD strain. Filled light grey regions indicate B10-derived DNA segment on chromosome 1. Filled black regions, on which three stars denote the location of the *Slc11a1* KO, indicate 129-derived DNA segment on chromosome 1. Filled dark grey regions indicate B6-derived DNA segment on chromosome 3. Open regions represent DNA segment between the in and out allelic markers at the boundaries. Lines delineate NOD-derived DNA. Vertical arrows designate the *Idd5.1*, *Idd5.2*, and *Idd5.3* regions. The diagram is to scale.



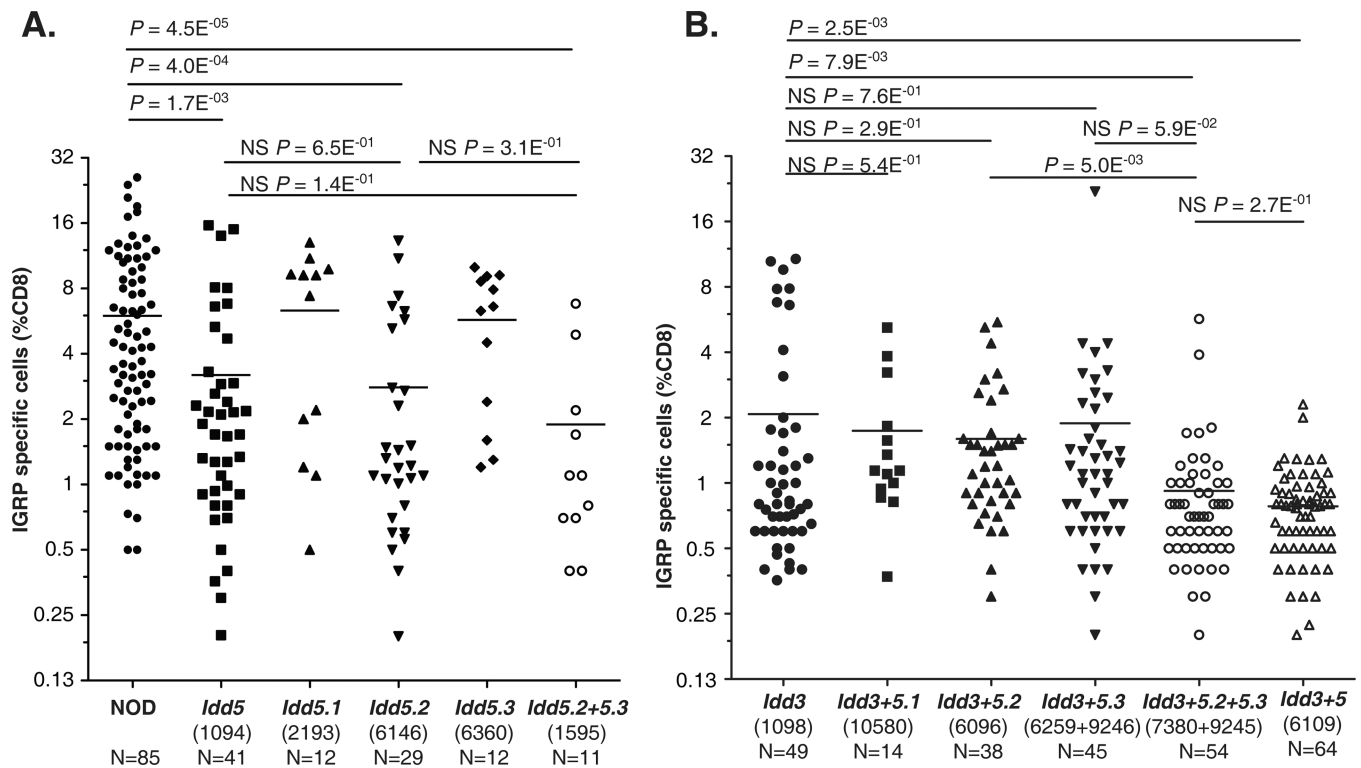
**Figure 2.** Diabetes frequencies of the novel congenic strains developed specifically for this study. The diabetes frequency studies represented in Figures 2A and 2D were performed at Merck Research Laboratories whilst the studies in Figures 2B, 2C, and 2E were conducted at Taconic. Group size N is indicated at the bottom of the genetic map of each strain. The *p*-values were calculated by comparing the Kaplan-Meier survival curves of the indicated strains using the log-rank test. NS, not significant, *p*-value  $> 0.05$ . (A) Diabetes frequencies of the *Idd5.2* congenic Lines 3388 and 6146 females were compared with NOD females. (B) Diabetes frequencies of the *Idd5.3* congenic Lines 6360 and 7345 females were compared with NOD females. (C) Diabetes frequencies of the *Idd5.2+5.3* congenic Lines 1595 and 7341 females were compared with NOD-like Line 6359 females. (D) Diabetes frequencies of the *Idd3* congenic Line 1098, the *Idd3+5.2* congenic Line 6096, and the *Idd3+5.3*

congenic Line 6259 females were compared. Note that the y-axis is truncated due to the low frequency of T1D in the strains examined. (E) Diabetes frequencies of the *Idd3* congenic Line 1098, the *Idd3+5.3* congenic Line 9246, two *Idd3+5.2+5.3* congenic Lines 7380 and 9245, the *Idd3/5* congenic Line 6109 females were compared. Note that the y-axis is truncated due to the low frequency of T1D in the strains examined.



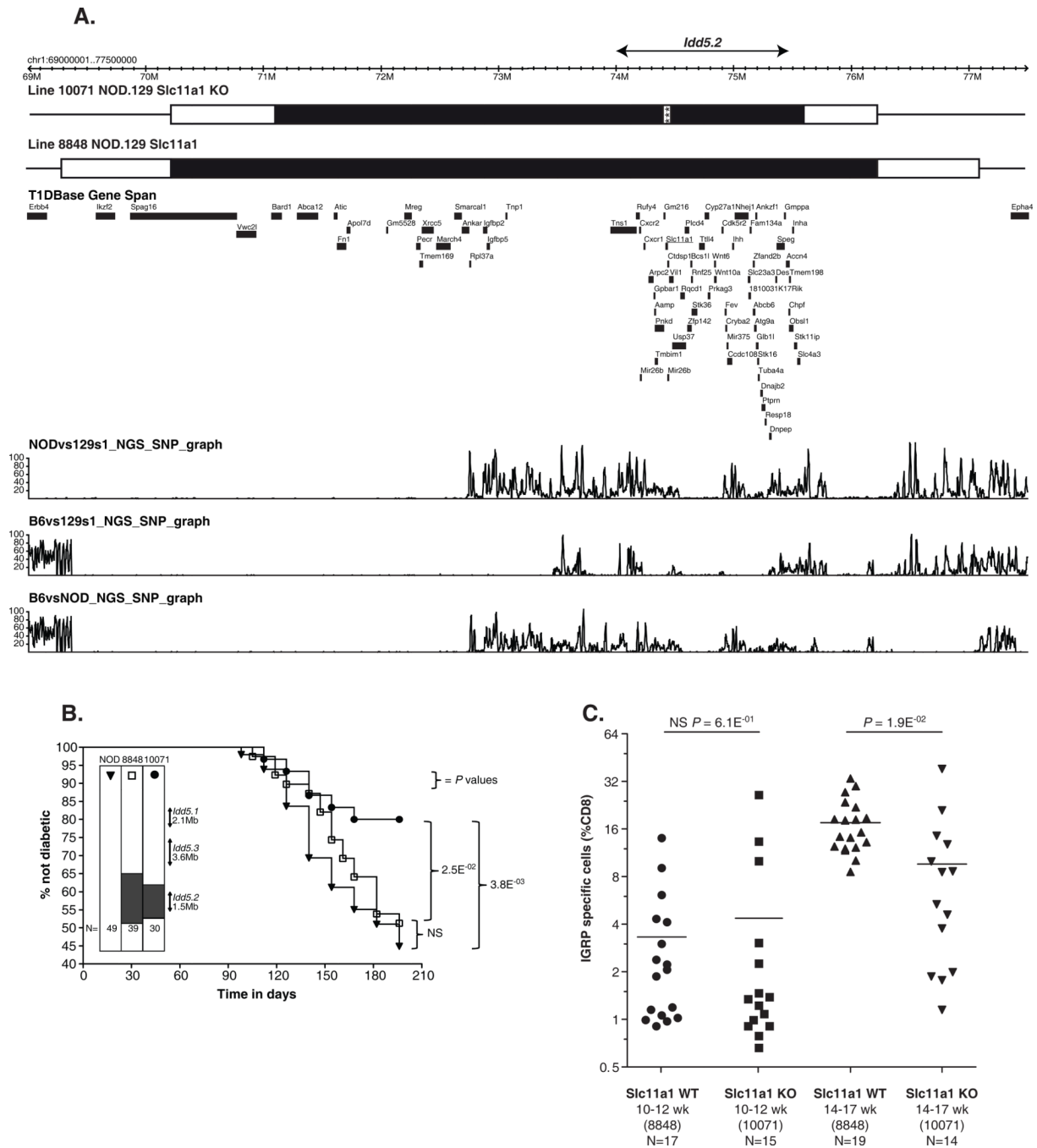
**Figure 3.**

A hierarchy in the insulinitis protection in *Idd3+Idd5*-sub-region(s) congenic strains. Non-diabetic females of the congenic *Idd3* (Line 1098), *Idd3+5.3* (Line 9246), *Idd3+5.2+5.3* (Lines 9245 and 7380), and *Idd3/5* (Line 6109) at 196 days of age were sacrificed and insulinitis scored. Each diabetic mouse was assigned an insulinitis score of '3' indicated by an open symbol. For each group, the number (X) of pancreases that have an average insulinitis score of zero was indicated on top of the x-axis and written as '+X zero', because of the Log<sub>2</sub> scale on the y-axis. Horizontal lines on the graph depict median values. Note that *Idd3/5* strain had a median of zero. Each pair of strains is compared using Mann-Whitney U test. NS, not significant, *p*-value > 0.05.



**Figure 4.**

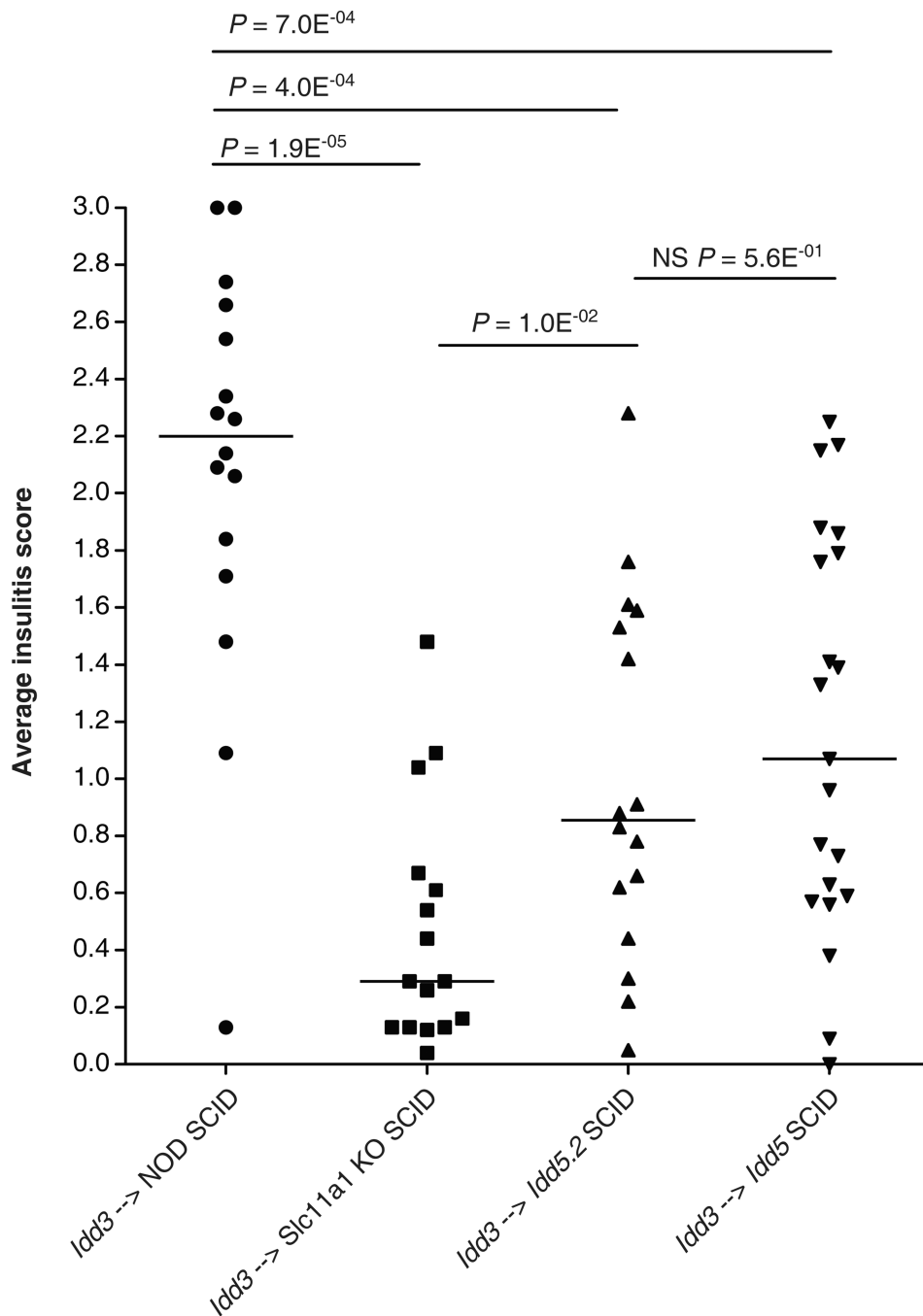
The contribution of *Idd3* and *Idd5* sub-regions to the restoration of CD8<sup>+</sup> T cell tolerance to endogenous islet antigen IGRP. Results were compiled from 13 independent experiments, each of which analyzed 6–12 female mice per strain, at 10–14 weeks of age. Female NOD, *Idd5*, *Idd5.1*, *Idd5.2*, *Idd5.3*, *Idd5.2+5.3*, *Idd3*, *Idd3+5.1*, *Idd3+5.2*, *Idd3+5.3*, *Idd3+5.2+5.3*, and *Idd3/5* mice were infected with Vac-K<sup>d</sup>IGRP. One week later, spleens were harvested, and analyzed by FACS for CD8<sup>+</sup> IGRP-tetramer<sup>+</sup> cells. 30 out of 91 NOD females, 18 out of 46 *Idd5* females, 29 out of 58 *Idd3* females, and 30 out of 71 *Idd3/5* females shown on the graph were published previously (30). These mice were included in the analysis because they were contemporaneous controls of other congenic strains that were examined at the same time. Horizontal lines depict the means. Pairs of strains were compared using unpaired t-test with Welch's correction. NS, not significant,  $p$ -value  $> 0.05$ . (A) *Idd5.2* alone is sufficient to partially restore CD8<sup>+</sup> T cell tolerance to self-antigen IGRP. (B) More than one *Idd5* sub-regions need to be combined with *Idd3* to further restore CD8<sup>+</sup> T cell tolerance to self-antigen IGRP.



**Figure 5.** *Slc11a1* KO strain is protected from T1D and shows partially restored CD8<sup>+</sup> T cell tolerance to endogenous islet antigen IGRP. (A) Genetic intervals of the *Slc11a1* WT and *Slc11a1* KO strains. Filled black region indicates the 129-derived DNA and white bars indicate the regions between the ‘in’ and ‘out’ markers. The gene content is displayed in the T1Dbase Gene Span tract (based on GRCm37 genome build), with genes shown in black and *Slc11a1* underlined. The SNP density tracks are based on the next generation sequencing (NGS) data and displays SNPs per 10 kb. (B) Diabetes frequencies of the NOD, the *Slc11a1* WT and the *Slc11a1* KO females were compared. The diabetes frequency studies were conducted at Taconic. (C) *Slc11a1* KO alleles contribute to the reduced percentage of self-reactive IGRP

specific CD8<sup>+</sup> T cells. Results were compiled from 6 independent experiments, each of which analyzed 6–8 female mice per strain, at either 10–12 weeks of age, or 14–17 weeks of age. Female *Slc11a1* WT and *Slc11a1* KO mice were infected with Vac-K<sup>d</sup>IGRP. One week later, spleens were harvested, and analyzed by FACS for CD8<sup>+</sup> IGRP-tetramer<sup>+</sup> cells. Horizontal lines depict means. Pairs of strains were compared using unpaired t-test with Welch's correction. NS, not significant, *p*-value > 0.05.





**Figure 6.**

*Idd5.2* or *Slc11a1* KO alleles expressed by non-lymphocytes in the host conferred insulinitis protection. Female NOD-SCID, *Idd5*-SCID, *Idd5.2*-SCID and *Slc11a1* KO-SCID mice were reconstituted with DC-depleted spleen and lymph node cells from *Idd3* female donor mice. Ten weeks after reconstitution, pancreata were harvested and H&E stained sections were assessed for insulinitis. Each diabetic mouse was given an arbitrary insulinitis score of '3'. Data are pooled from 7 independent experiments. Horizontal lines depict median values. Pairs of strains were compared with the Mann-Whitney U test.