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Snail transcription factors in hematopoietic cell development: a model of functional redundancy

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Abstract

Coordinated gene expression is crucial in facilitating proper lymphoid cell development and function. The precise patterns of gene expression during B and T cell development are regulated through a complex interplay between a multitude of transcriptional regulators, both activators and repressors. We have recently identified the Snail family of transcription factors as playing significant and overlapping roles in lymphoid cell development in that deletion of both *Snai2* and *Snai3* was required to fully impact the generation of mature T and B cells. Analyses using compound heterozygote animals further demonstrated that *Snai2* and *Snai3* were partially haplo-sufficient and relatively equivalent in their ability to preserve B cell generation in the bone marrow. In this review, we summarize studies elucidating the role(s) of the Snail family in hematopoiesis with a focus on lymphoid cell development. Using the Snail family as an example, we discuss the concepts of functional redundancy and strategies employed to assay transcription factor families for “intra-member” compensation.

Keywords

Snail; transcription factor; hematopoiesis; B cell; lymphocyte; redundancy; leukemia

An introduction to the Snail family

Embryonic development along with the development of cell lineages with specialized functions requires a properly orchestrated network of gene expression. The Snail family is a primordial metazoan collection of transcriptional regulators consisting of three members: Snai1 (Snail), Snai2 (Slug) and Snai3 (Smuc) [1, 2]. Snail family members classically function as transcriptional repressors. To carry out this function, all three members share

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two cardinal features. Within the C-terminal portions of each protein reside multiple C_2H_2-type zinc finger DNA-binding domains (DBD). Snai1 possesses four DBDs while both Snai2 and Snai3 possess five [3]. These DBDs recognize the consensus E-box sequence, CANNTG. In particular, Snail proteins show a strong preference for GC-rich central dinucleotides [4]. At the extreme N-terminus, each protein contains a SNAG domain (Snail/Gfi1) [5]. Using this domain, the recruitment of various chromatin co-repressor modifiers leads to the generation of a transcriptionally silent state. Examples of such modifiers include histone deacetylaseses, such as HDACs 1/2, lysine-specific demethylases (LSD1) and methyltransferases (EZH2) [6, 7].

The Snail family has been demonstrated to participate in a wide variety of physiological and pathological processes [8, 9]. The founding member, *Snai1*, was first described in *Drosophila melanogaster* and was shown to be essential in the developing embryo for proper ventral-dorsal patterning leading to eventual mesoderm formation [10, 11]. Analogous to *Drosophila*, deletion of murine *Snai1* results in embryonic lethality due to gastrulation defects [12]. This points to an evolutionarily conserved role for *Snai1* in the developing embryo. Deletion of *Snai1* at the epiblast stage also resulted in embryonic lethality, in this instance due to global defects in vascularization [13]. Continuing to focus on the murine system, deletion of *Snai2* did not result in organismal catastrophe. *Snai2* germline knockout mice possess impaired physical and hair follicle developmental kinetics most readily observed within the pre-weaning period [14, 15]. On select genetic backgrounds, these mice develop piebaldism (suggestive of defective melanocyte function) and symptoms analogous to Type II Waardenburg syndrome (characterized by hearing loss and skin/hair pigment anomalies) [16]. Of significance, a functional redundancy between Snai1 and Snai2 in both chondrogenesis and cranial-facial development has been previously shown [17, 18]. Recently, two laboratories including our own have described the generation of *Snai3* deficient animals. Unlike both *Snai1* and *Snai2*, no phenotypes were apparent upon the deletion of only *Snai3* [19, 20]. Our studies, however, additionally analyzed the loss of Snai3 in the context of a Snai2 deficient animal, a germ line double knockout (DKO), which resulted in clear developmental abnormalities [20]. Some of these included severe “runting” with an overall failure to thrive and a definitive skewing towards generation of the male sex. Additionally, multiple lymphopoietic abnormalities were apparent only upon deletion of both genes (discussed below). This data supported a physiological role for *Snai3* along with a continued theme of functional redundancy among various Snail members.

The Snail family and hematopoiesis

At this time, there is no data elucidating the role of *Snai1* within the hematopoietic system. Recently, we have generated a hematopoietic-specific deletion of *Snai1* via utilization of the *Vav-Cre* deleter strain and a strain possessing a conditionally targeted *Snai1* gene. Unlike embryogenesis, *Snai1* is not required for hematopoiesis since these conditional *Snai1*-deleted mice are viable, outwardly healthy, and with no obvious hematopoietic deficiencies (unpublished data). A more detailed analysis of these mice is underway.

For the rest of this article, we shift our focus towards the hematopoietic functions of both Snai2 and Snai3. In an initial report, Inukai et al. demonstrated overexpression of Snai2 in

several human B cell leukemia cell lines. Upregulation of *Snai2* was dependent upon the E2A-HLF oncoprotein generated from a t(17;19) chromosomal translocation. Usage of the murine IL-3 dependent Baf3 Pro-B cell line demonstrated that overexpression of Snai2 was sufficient to confer resistance to apoptosis induced by growth factor withdrawal which was accompanied by exit from the cell cycle [21]. In regards to cancer progression, Snai2 and Snai1 are most commonly identified for their ability to induce epithelial-to-mesenchymal transition (EMT) resulting in a more migratory and invasive cancer phenotype. This result suggested an alternative mechanism for the survival of transformed cells. Less appreciated, but maybe just as significant; these data may point to a role for Snai2 in chemotherapeutic resistance. This is most relevant for DNA damaging agents such as Doxorubicin, which are most effective in actively cycling cells. Interestingly, Perez-Losada et al., demonstrated the ability of c-Kit signaling to induce *Snai2* expression. *In vitro* studies utilized both Baf3 and LAMA-84 cells overexpressing c-Kit. Of note, LAMA-84 cells were originally derived from a chronic myeloid leukemia (CML) patient undergoing blast crisis [22]. The mechanism of *Snai2* upregulation becomes relevant when one considers that c-Kit is highly expressed on the surface of acute myeloid leukemia (AML) cells [23, 24]. Unfortunately, this study did not conduct any experiments to assess the downstream consequences of *Snai2* induction in LAMA-84 cells. Overall, these data provided some interesting insights into the potential role(s) of the Snail family in promoting hematological malignancies.

Moving forward, the point of emphasis shifted towards the role of Snai2 in more physiologic hematopoietic settings. Included within the Perez-Losada report was an initial description of hematopoiesis in the *Snai2* knockout mouse. They observed multiple defects, which mainly focused on erythropoiesis [14]. Complete blood counts showed a trend towards lower erythroid “output”. Using *in vivo* models of phenylhydrazine (PHZ)- and pregnancy-induced anemia, a lower percentage of $Ter119^{+}$ cells was observed in the spleen. This pointed to a role for *Snai2* in hematopoietic stress responses and its requirement in reconstituting the erythroid lineage. Intriguingly, $Snai2^{+/-}$ animals showed similar defects as the $Snai2^{-/-}$ suggesting a gene dosage component. Examination of steady state hematopoiesis did not show any derivation from normal myeloid and B cell generation. However, a decreased ratio of developing CD4 and CD8 double positive thymocytes was observed in *Snai2* knockout animals. This was apparently a result of increased apoptosis as assayed by TUNEL staining of thymic cross-sections. Almost immediately following this publication, a separate study from the Look lab showed the expression of *Snai2* in multiple bone marrow progenitor lineages including the hematopoietic stem cell (HSC), both long term (HSC-LT) and short term (HSC-ST), common lymphoid (CLP) and common myeloid (CMP) progenitors among others. Colony forming assays were performed to evaluate differentiation potential of progenitors in the bone marrow and spleen. While the $Snai2^{-/-}$ progenitors trended towards an increase in colony forming units (CFUs) for various lineages, no clear-cut enhancement over wildtype (WT) was observed. Expanding on this, they assayed the ability of $Snai2^{-/-}$ animals to reconstitute the hematopoietic compartment following an LD_{50} dose of total body irradiation (TBI), also a stress response [25]. By 13 days post-irradiation, all $Snai2^{-/-}$ animals had died due to pancytopenia. This was in stark contrast to both WT and $Snai2^{+/-}$ animals in which approximately fifty percent survival was achieved when followed out to 29 days. Closer examination of these animals demonstrated increased apoptosis within the

Snai2$^{-/-}$ lineage negative (Lin^{-}) bone marrow leading to the pancytopenia. As such, administration of a thrombopoietin analog rescued irradiation-induced lethality of *Snai2*$^{-/-}$ animals. Using bone marrow chimeras, a follow up study demonstrated that loss of *Snai2* in the hematopoietic compartment was sufficient to propagate the irradiation phenotype described above [26]. Importantly, WT bone marrow administered to a *Snai2*$^{-/-}$ host completely prevented any lethality providing further evidence for a hematopoietically cell intrinsic role for *Snai2* that was independent of the surrounding bone marrow stroma. Using a well-designed combination of knockout and transgenic animals, it was shown that Snai2 functioned downstream of p53 to block the activation of Puma, a pro-apoptotic BH3-only family member. This was accomplished via a direct E-box interaction within intron 1 of the *Puma* gene. Somewhat surprisingly, this effect was specific to Puma as *Snai2*$^{-/-}$ *Puma*$^{-/-}$ compound knockout animals were completely radio-resistant. If other apoptotic mediators were playing a role, an intermediate phenotype would have been expected. Finally, a group led by Wen-Shu Wu examined the self-renewal capacity of hematopoietic stem cells with and without *Snai2* [27]. Using limiting dilution bone marrow chimera transplants, it was determined that *Snai2*$^{-/-}$ marrow possessed an approximately 8-fold higher repopulation efficiency as compared to *Snai2*$^{+/-}$. Competitive serial transplantation, a gold standard for assessing HSC fidelity, further demonstrated the enhanced ability of the *Snai2*$^{-/-}$ HSC to reconstitute the entire hematopoietic system. Incorporation of 5-ethynyl-2'-deoxyuridine (Edu) combined with Annexin V cell surface staining demonstrated this result was a consequence of higher proliferative capacity rather than augmented apoptosis within the *Snai2*$^{-/-}$ Lin^{-} Sca1^{+} compartment. This result coalesces with the previously identified ability of Snai2 overexpression to induce cell cycle exit by Baf3 cells [21]. While the molecular targets of Snai2 were not identified, they may be similar to what has been previously observed for Snai1. In a non-tumorigenic context, Snai1 is able to inhibit the cell cycle of MDCK cells by repressing cyclin D2 at the transcriptional level [28]. In a non-canonical role for the Snail family, it was also reported that Snai1 co-associated with EGR-1 and SP-1 to upregulate p15INK4b leading to the arrest of HepG2 cells [29].

The function of the Snai3 transcriptional regulator in the hemaotopoietic system was first analyzed in an indirect investigation, the analysis of binding proteins to negative regulator elements of the murine *Itgb2l* (Pactolus) gene [30]. Using a combination of EMSA and supershift assays, it was shown that Snai3 was capable of binding to these transcriptional regulatory sites. The most intriguing aspect of this work was the hypothesis that in scenarios where diverse cell types, such as B cells and neutrophils, express a common transcriptional activator (e.g. PU.1), differential expression of a negative regulator may potentially augment lineage specific expression of downstream genes. Thinking more globally, Dahlem et al. asked whether retroviral-induced overexpression of *Snai3* in a bone marrow chimera model was capable of impairing hematopoiesis. Using c-Kit and Sca-1 based analysis of lineage depleted marrow, it was determined that overexpression of *Snai3* did not alter ratios of various subsets of hematopoietic progenitors [31]. However, inspection of peripheral blood demonstrated a clear loss of B and T cell lineages in cells that were infected with the *Snai3*-expressing retrovirus (as assayed by GFP expression). This effect was dose dependent as B and T cell populations were present when Snai3 overexpression was at a modest amount (GFP low). Importantly, B and T cells were present when the empty vector was expressed at

both low and high levels arguing against any retroviral-induced artifacts. These data suggested that Snai3 was either capable of saturating endogenous E-boxes necessary for the proper bifurcation of lymphoid and myeloid lineages or directly repressed gene expression by the recruitment of transcriptional control complexes. This data also alluded to a requirement for precise regulation of *Snai3* within the hematopoietic system.

Phenotypic analysis of animals lacking both Snai2 and Snai3 transcription factors

Two groups recently described the deletion of the *Snai3* gene using both conditional and germ line deletion models. Upon deletion of *Snai3*, no effects were observed in any hematopoietic lineage assayed [19, 20]. Indeed the *Snai3*$^{-/-}$ strains created by both groups displayed little to no phenotypic changes from WT. Gene expression analysis of *Snai1*, *Snai2* and *Snai3* in bone marrow derived lineages demonstrated widespread *Snai1* expression in T and B precursor and mature cells, as well as cells of the myeloid lineage[20]. *Snai2* expression was more limited with highest levels of transcripts found in immature bone marrow cells (B220^{-}, CDllb^{-} cells) and double negative (DN) (CD4^{-} CD8^{-}) T cells of the thymus. *Snai3* expression was highest in the DN, the double positive (DP) (CD4^{+}CD8^{+}) and CD8^{+} cells of the thymus and spleen. The potential of the Snail family of proteins to functionally complement one another led us to create and analyze a germ line DKO animal lacking both the *Snai2* and *Snai3* genes [20] (the absence of *Snai1* in the germ line creates an embryonic lethality) [13]. In this animal we observed multiple hematopoietic and non-hematopoietic phenotypes, all of them greater than that found in the single *Snai2*$^{-/-}$ or *Snai3*$^{-/-}$ animals. The DKO animals were distinctly smaller than the single deficient animals and had significantly smaller spleens and thymi, which possessed morphological distortions. The most striking hematologic phenotype of the DKO animals was a loss of B cells in the bone marrow. This was correlated with an expanded myeloid compartment. In the DKO thymus, double positive thymocytes were reduced in favor of enhanced percentages of CD4 single positive T cells. Unlike in the marrow, skewing of thymocyte populations displayed variable penetrance. Analysis of compound heterozygotes suggested that on a per allele basis, one allele of either *Snai2* or *Snai3* equivalently compensated for the loss of the other three alleles. The DKO animals did possess a normal *Snai1* gene; which is transcriptionally active in immature B and T cell subsets. As such, incomplete complementation of the DKO animal via Snai1 may have continued to mask the full dependence of hematopoietic cell development on members of the Snail family.

One of the unanswered questions from our recent study was the level of stromal contribution to the phenotypes observed. Development of both B and T cells is dependent upon proper organization within their respective sites of generation. For example, pre-Pro-B and Pro-B cells congregate in bone marrow niches high in CXCL12 and IL-7, respectively [32]. In contrast, Pre-B cells were not found to associate with either zone suggesting precise localization is required for the proper progression along each stage of B cell maturation. Histological examination of the germline *Snai2* and *Snai3* DKO bone marrow assessing B cell localization has not been performed. However, as mentioned above, the analysis of thymic cross-sections from DKO mice showed gross alterations in overall architecture [20].

In DKO mice, intense hematoxylin staining was observed in the region corresponding to the thymic medulla, the site of single positive thymocyte negative selection. Normally, the thymic cortex demonstrates the highest intensity of hematoxylin staining due to high numbers of CD4 and CD8 double positive thymocytes. Since double positive thymocytes were still the majority population in the DKO thymus, this argues against preferential staining of an over expanded single positive compartment. Rather this may suggest a dysfunctional organization of the thymus due to altered chemokine signaling. Multiple studies have demonstrated the importance of stromal-derived CCL25 and P-selectin in thymic homing and organization [33, 34]. Interestingly, competitive bone marrow chimeras using WT and *CCR9*$^{-/-}$ donor marrow revealed deficiencies in *CCR9*$^{-/-}$ thymocyte development that mirrored many of the phenotypes observed upon thymic stromal-specific deletion of *Il7* [35]. This points to a role for CCL25 not only in thymic homing, but perhaps also in positioning developing thymocytes in the proximity of survival/proliferative signals such as IL-7. Given the known role of the Snail family in cellular migration (i.e. gastrulation and EMT), it is possible that the contributions of this family to lymphocyte development include a role in lymphocyte migration. This function would assist in orchestrating lymphocyte maturation by allowing a cell at any given developmental stage to receive signals required for its survival and continued differentiation. Due to the lack of an animal model possessing a *Snai2* conditional allele, the examination of stromal contributions in providing organizational and/or survival signals will require the use of bone marrow chimera models. While inconvenient due to such factors as length of time for functional reconstitution, these studies may provide the most flexibility in assaying the stromal versus hematopoietic function of each Snail gene. In this sense, one has the option of “selectively” deleting *Snai2* in the stroma while also deleting *Snai3* in just the hematopoietic compartment and *vice versa*. Experiments such as these would be virtually impossible using current recombinase deleter strains.

Functional redundancy within the Snail gene family

The requirement of *Snai2* deletion to unveil a role for *Snai3* in hematopoiesis was somewhat of a surprise. Classically, the thought has been: delete *Gene X*, deduce the function of *Gene X* based on the resulting phenotype(s). Clearly, nature does not operate in such a simple and linear fashion especially when considering multigene families of transcription factors. So can the need for compound knockouts be predicted? In reality, the answer is most likely not a simple yes or no. When dealing with a multigene family such as Snail, one might transcriptionally profile the cell/tissue type of interest for overlapping expression. While coexpression of multiple Snail members may suggest a potential for redundancy, it does not rule out of the possibility of completely independent functions for each factor within a given cell. Conversely, a situation lacking coexpression rules out nothing. This was demonstrated for *Snai1* and *Snai2* during chondrogenesis [18]. In the steady state, *Snai1* and *Snai2* were expressed mutually exclusively within hypertrophic and proliferating chondrocytes, respectively. However, deletion of *Snai1* resulted in expression of *Snai2* in hypertrophic chondrocytes and *vice versa*. It was later shown that Snai1 and Snai2 could repress one another at the level of transcription [36]. In a circumstance such as this, analysis of single knockouts provided the evidence for a compound knockout approach.

Within hematopoiesis, functional redundancy among transcription factors is not a new concept. For example, the GATA family of transcriptional regulators possesses six members, three of which (*GATA1-3*) are highly expressed in hematopoietic lineages. Using mouse deficiency models, the lack of both *GATA-1* and *GATA-2* has a more profound impact upon primitive hematopoiesis during development than seen with animals lacking only a single such factor [37]. Additionally, all three Runx family members (*Runx1*, *Runx2* and *Runx3*) are expressed in the hematopoietic stem cells and may play redundant roles in influencing stem cell quiescence and cycling [38].

Functional redundancy for transcription factors implies common gene target recognition. However unique protein sequences present within highly conserved family members can alter target site specificity by recruiting additional targeting factors, as demonstrated by the highly conserved members of the ETS family [39].

Once a functional redundancy is validated using compound knockouts, the next question becomes the mechanism. The optimal way to assess this phenomenon would be to determine the transcriptional footprint of each factor involved through the combined use of ChIP- and RNA-Seq. ChIP-Seq will identify direct transcriptional targets while RNA-Seq of knockout animals will additionally identify indirect genetic interactions. In the case of transcriptional repressors, genes downregulated in a knockout would most likely represent an indirect regulation (i.e. derepression of another repressor). While seemingly arduous, this approach is much more globally informative and unbiased compared to the examination of predetermined gene targets. For both forms of analyses, WT and knockout animals are required. Wildtype animals become extremely valuable in their ability to tell us what happens normally (e.g. Snai2 versus Snai3 bound genes). Analysis of single and compound knockouts will determine the genetic extent of redundancy and may even narrow the candidate list of “essential” targets. Consider the following hypothetical (and over-simplified) example, Snai3 binds the *Gene X* and *Gene Y* promoters in WT Pre-B cells (Figure 1A). Upon loss of Snai3, Snai2 now binds only the *Gene X* promoter. As such *Gene X* is still repressed but now expression of *Gene Y* is enhanced with no resultant phenotype (Figure 1B). Upon deletion of both *Snai2* and *Snai3*, *Gene X* is now also transcriptionally enhanced and a B cell phenotype is now observable (Figure 1C). This result would identify two things: 1) common and specific targets for each Snail member and 2) genes potentially responsible for phenotypes observed in the compound knockout. Of course, these scenarios become more complex as the family being studied and their list of *in vivo* targets grow. One factor not being discussed is how dysregulated expression of known post-translational modifiers (PTM) may augment the system being studied. This may be most relevant for factors under the influence of a multitude of PTM modalities.

Conclusions

To summarize, the Snail family consists of three evolutionarily conserved members. These factors have been most extensively studied in the fields of developmental and cancer biology. The examination of how this family regulates various aspects of immunology has been minimal at best. It is the opinion of the authors that the role of the Snail family in hematopoiesis will prove to be much more expansive than initially realized. Part of this

reasoning is based on the recent discovery that demonstrated a functional redundancy between *Snai2* and *Snai3* during lymphocyte development. This data also points to the evolutionary conservation of all three Snail members as a potential fail-safe mechanism allowing the fidelity of hematopoiesis in the face of single gene loss (i.e. germline or somatic mutation). An even more provocative question now becomes: how many functions of a particular gene have not been discovered due to redundancy with another family member? To completely unveil the functions of a specific gene product may ultimately require the generation and analysis of various single and compound knockout combinations.

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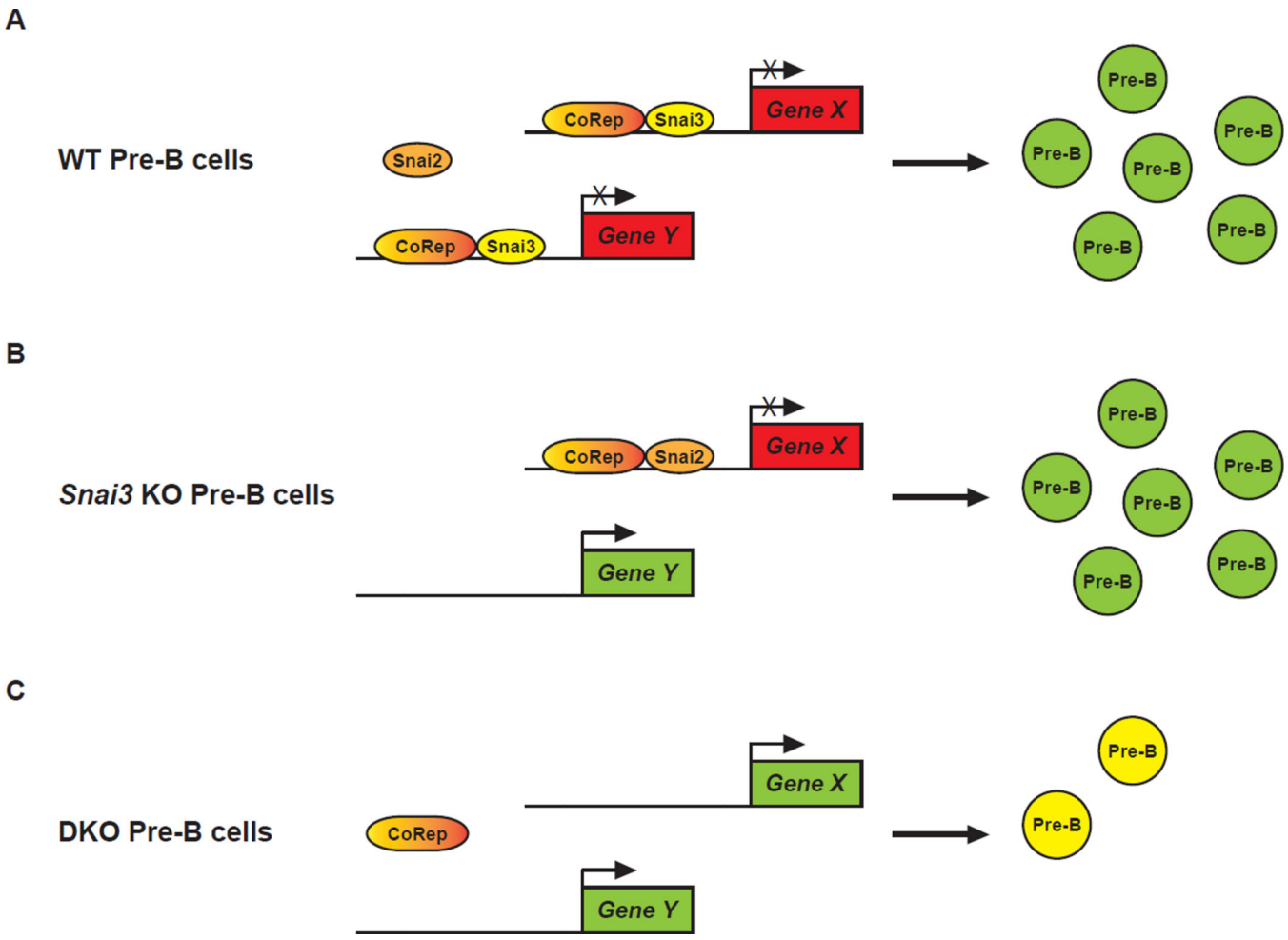

Figure 1. Redundancy model illustrating potential Snail compensation in lymphocyte development

(A) In a WT developing Pre-B cell, Snai3 inhibits transcription of its specific hypothetical target genes (*X* and *Y*) (red boxes) along with a Co-repressor protein (CoRep) allowing for normal development of Pre-B cells. (B) In the absence of Snai3, the Snai2 protein can facilitate the transcriptional repression of *Gene X*; however, it lacks the specificity to similarly repress *Gene Y* transcription (green box). The presence of the *Gene Y* protein product does not appreciably influence the development of the Pre-B cells. (C) The absence of both Snai2 and Snai3 allows for the transcription of both *Gene X* and *Gene Y* that results in the inhibition of the development of functional Pre-B cells (yellow cells).