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Mutagenesis Analysis of *ABCB8* Gene Promoter of *Danio rerio*

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ABSTRACT

The *ABCB8* is one of the members under the ABCB subfamily of ATP-Binding Cassette (ABC) transporter which possess the ability in regulating the intracellular iron and heme transport. The loss of function mutation of *ABCB8* gene leads to iron and heme accumulation in the cell which is highly toxic to human. However, the information regarding the expression regulation of this gene remains scarce. Hence, the objectives of this project are to determine the transcription factors binding site (TFBS) of *ABCB8* and to identify the transcriptional roles of the *cis*-elements through mutagenesis analysis. To examine this, total genomic DNA was extracted from *Danio rerio* and the promoter sequence was isolated by using specific pair of primers through polymerase chain reaction (PCR). The sample was sent for DNA sequencing and the result showed 98% similarities to the zebrafish DNA sequence from clone DKEYP-87A6 in linkage group 24. Besides, the TFBS was studied in aspect of TFBS abundance, TFBS composition and TFBS distribution. The two most abundant TFBSs based on liver-specific profile were HNF-3β and C/EBPβ, with 38 and 39 binding sites, respectively. The sequence of *ABCB8* promoter gene was mutated through substitution of the AP-1 binding site at location 535 with other nucleotides by using a pair of mutagenic primers (forward primer: 5'-TGGGGGTTTAGATATTGAAAC-3'; reverse primer: 5'-AACTCGC ATACATTTCAGTCATC-3'). This result may benefit the development of new diagnostics and therapeutics for iron-associated disorder.

Keywords: ABC transporter, *ABCB8* promoter sequence, *Danio rerio*, mutagenesis, TFBS

INTRODUCTION

Iron is major element that is involved widely in the metabolic process of human such as transportation of oxygen, synthesis of deoxyribonucleic acid (DNA) and transportation of electron (Dawson, 1988). In human body, iron is found mainly as a cofactor which is bound with the protein to form heme compound such as haemoglobin and myoglobin (Chiabrando, Vinchi, Fiorito, Mercurio, & Tolosano, 2014). Heme compound is important in cardiovascular physiology whereby it is involved in gaseous exchange and mitochondrial energy production. Besides that, it is also commonly associated with the oxygen transportation. Although heme is vital in biological process, but excess of heme compound is highly toxic to human body (Chiabrando *et al*., 2014).

ATP-Binding cassette transporter (ABC) is a huge superfamily which is an example for ATP-dependent pump (Vasiliou, Vasiliou, & Nebert, 2009). ABC transporters that encode membrane proteins are important in exporting and importing wide varieties of substrates and molecules across the membrane (Seguin & Ward, 2018). One of the subfamilies that branched out from ABC superfamily is ATP-Binding Cassette Subfamily B Member 8 (*ABCB8*). According to Vasiliou *et al*. (2009), *ABCB8* gene is vital in transportation of heme, phospholipids or intracellular peptides across the membranes in human.

Transcription factors (TFs) are proteins which consist of the DNA-binding domain that recognizes specific DNA sequence within the promoter region. This feature impacts the transcription regulation of the gene by binding to specific regions of the gene (Gonzalez, 2016). The processes that identify the cell types and developmental patterns are also controlled by the TFs. Transcription Factor Binding Site (TFBSs) analysis is an important way to understand the abundance, composition and the position of the TFs in the promoter region.

To date, the *ABCB8* can be found in many organs such as brain, eye, gill, intestine, muscles, and skin as

detected in teleosts, mice, human and primates (Lim *et al*., 2018a). Although the gene is expressed in many organs, information regarding the expression regulation of this gene remains scarce. It has been identified to be one of the main regulators in heme accumulation within the targeted cells, the exact molecular mechanism has not been studied thus far. Previous studies have indicated that transcription factors such as Sp1, NFY, p53 are all implicated in ABC transporter regulation in general (Seguin & Ward, 2018; Vasiliou *et al*., 2009).

Previous literature has been focusing on the gene functional characterization of *ABCB8* on zebrafish (Newman, Hin, Pederson, & Landelli., 2019). Lim, Chung, Ishak and Waiho (2021) have recently examined zebrafish promoters *ABCB4*, *ABCC1* and *ABCG2* via microinjection coupled with green fluorescence protein detection on zebrafish embryos, but none has place the spotlight on the *ABCB8* promoter to the best of our knowledge, albeit many have proven that gene orchestrators like enhancer and promoter have substantial roles in determining whether and when a gene will be activated (Hernandez-Garcia & Finer, 2014; Lim, Chung, Chong, & Lee, 2018b; Lim, Chung, Chong, & Lee, 2019a; Lim , Chung, Chong, & Lee, 2019b; Liu & States, 2002; Mishra, Dhanda, Siwach, Aggarwal, & Jayaram, 2020; Wang, Cheng, Li, Wu, & Zhao, 2018). The verification of the involvement of these transcription factor in feedback regulation of *ABCB8* needs to be determined by using the site-directed mutagenesis. Therefore, this study is to determine the TFBSs and the roles of these *cis*-elements through mutagenesis. Through mutagenesis, it is expected that the gene expression of the *ABCB8* gene in zebrafish will be inhibited. This study forms the foundation to the development of new diagnostics and therapeutics for iron-associated disorder in human, especially when knockout experiments on this zebrafish promoter and its mutant show promising outcomes in the future.

MATERIALS & METHODS

Total Genomic DNA Extraction

The fish care and fish tissue collection were performed following the authorization and standard operating procedures set by the Animal Ethics Committee, Universiti Malaysia Sarawak (UNIMAS/TNC(PI)-04.01/06-09(17)). The whole *D. rerio* was sliced finely on ice and transferred into 1.5 mL of microcentrifuge tube prior to the total genomic DNA extraction procedures emulating that of Chung (2018) as well as Lim, Chung, Lau, Aziz and Gan (2021).

Gradient PCR

A pair of primers for *ABCB8* of *D. rerio* (forward primer: 5'-ATG GTA CCG TTA AAT CAA GGA CAA GCG T -3'; reverse primer: 5'-ATG AGC TCA GAA ACG ACA GAG TGA TGA AA -3') were optimized in terms of annealing temperature using T100^{TM} Thermal Cycler (BIO-RAD, USA), procedures were done as described by Lim, Chung, and Hasnain (2020) with a few amendments. The optimal temperature of the primer was calculated and then the temperature obtained was applied for the following PCR reaction. Next, 10 µM *ABCB8* forward and reverse primer were diluted before continuing with the preparation of the PCR master mixtures. A 10x dilution was performed to obtain a 10 µM primer solution from the 100 µM of primer stock. The PCR master mixtures solution was prepared by adding 10X *EasyTaq* buffer with (Mg^{2+}), 2.5 mM dNTPs, 50ng/µL zebrafish genomic DNA, 2.5 units *EasyTaq* DNA Polymerase, 10 µM *ABCB8* forward and reverse primer respectively, with the remaining volume topped up with double distilled water. Then, 20 µL of master mixture was transferred into each PCR tube. After that, the mixture was subjected to thermal cycling for 35 cycles. The cycling conditions were initial denaturation at 95˚C for 3 mins, denaturation at 95˚C for 35 sec, annealing between 58˚C to 65˚C for 30 sec and extension at 72˚C for 1 min 30 sec. Agarose gel electrophoresis was done after the PCR to check on the PCR products.

Restriction digestion and cloning

The restriction digestion mixtures for digesting pGEM-T and pGL 3.0 vectors were prepared separately. The digesting mixture for pGEM-T mixture was prepared by adding 4 µL of DNA (0.5-1.0 µg/µL), 2 µL of 1X Tango Buffer, 2 µL of *KpnI* restriction enzyme, 1 µL of *SacI* restriction enzyme, and 11 µL of nuclease-free water. While the digesting mixture for pGL 3.0 vector was prepared by adding 10.2 µL of DNA (0.5-1.0 µg/µL), 2 µL of 1X Tango Buffer, 2 µL of *KpnI* restriction enzyme, 1 µL of *SacI* restriction enzyme, and 4.8 µL of nuclease-free water.

The mixture was incubated at 37˚C overnight, followed by incubation at 80˚C to inactivate the enzymes. The inactivate mixture was analysed on 1% agarose gel electrophoresis at 90 V for 40 minutes. The cloning process was conducted in accordance to that portrayed by Jee *et al*. (2017).

Transcription factor binding site (TFBS) analysis

The consensus sequence was used to perform transcription factor binding site (TFBS) analysis by using MATCH tool to determine the TFBS abundance, TFBS composition and TFBS distribution (Kel *et al*., 2003). The overall TFBS was identified by inserting the *ABCB8* sequence as an input and the TFBS frequency per nucleotide was calculated and recorded.

Mutagenic primer design and synthesis

Mutagenic forward and reverse primers for *ABCB8* were designed, emulating that of Yeaw, Lim and Chung (2020) as well as Md Yusni, Lim and Chung (2020). The AP-1 binding site was mutated by substituting the nucleotide in core binding site with another nucleotide. After that, suitable forward and reverse primers of 20 to 25 mers were selected on the region of the conserved domain. The primers were screened for melting temperature, self-complementary, presence or absence of secondary structure and GC content by using the OligoCalc tool. After that, the selected primers order was sent to First BASE Laboratories for primer synthesizing.

Site-directed mutagenesis

Mutagenesis experiment was conducted by using KOD Plus Mutagenesis Kit (Toyobo, Japan) according to the manufacturer's protocol. The mutagenesis reaction mixture was prepared by adding 10× buffer for iPCR, 2 mM dNTPs, 50 ng/ μL pGL 3.0 with mutated *ABCB8* insert, 1 μL KOD -Plus- DNA polymerase, 10 pmol/ μL *ABCB8* mutagenic forward and reverse primer respectively, with the remaining volume topped up with PCR grade water. After assembling the mixture, the reaction mixture was subjected to a PCR cycle for 10 cycles. The cycling parameters for inverse PCR were initial denaturation at 94˚C for 2 min, denaturation at 98˚C for 10 sec, followed by primer annealing between 45˚C and 52.3˚C for 30 sec, extension at 68˚C for 7 min and holding at 12˚C.

Following the PCR cycle, 2 μL of *Dpn* I restriction enzyme (10 U/μL) was added to the 50 μL PCR reaction mixture. After that, the mixture was mixed thoroughly by pipetting and incubated at 37˚C for 1 hour to digest the plasmid. Then, a total of 15 μL ligation mixture was prepared by adding 2 μL of *Dpn1* treated PCR product, 7 μL of ddH_2O, 5 μL of ligation high and 1 μL of T4 Polynucleotide kinase. Next, the DNA that was treated with 1 μL of *Dpn* I was transformed into the XLI-Blue *E. coli*. The transformation reaction mixtures were plated out on LB-ampicillin agar plates and incubated at 37˚C for 16 hrs.

RESULT

TFBS analysis

The *ABCB8* promoter gene sequence was analysed by using MATCH tool to identify the overall TFBS frequency per nucleotide. Based on the result obtained from MATCH tool, the frequency of sites per nucleotides based on liver-specific profile is 0.099785.

$$\text{Frequency of sites per nucleotide} = \frac{139}{1393} = 0.099785$$

The composition of the transcription factor binding sites were identified based on liver-specific profile. Based on Figure 1, there are 12 TFBSs detected on the promoter region of *ABCB8* gene. The TFBSs detected were GATA-3, HNF-1, HNF-3β, C/EBPβ, AP-1, CHOP-C/EBPα, USF, HNF-4, USF, CREB, YY1, NF-1 and TATA. Based on the data obtained, C/EBPβ had the highest amount which hits 39 binding sites in the promoter region.

The TFBS distribution of the 1.5kb promoter region of *ABCB8* was analysed at every 100 bp and each of the TFBSs were plotted in the Figure 2. Based on the Figure 2, the 12 TFBSs detected in the promoter sequence (GATA-3, HNF-1, HNF-3β, C/EBPβ, AP-1, CHOP-C/EBPα, USF, HNF-4, USF, CREB, YY1, NF-1 and TATA)

were plotted in a 100 bp interval format. The C/EBPβ and HNF-1 clustering is one noticeable observation on the distribution pattern of these TFBSs, while the other TFBSs were found scattered across the promoter region.

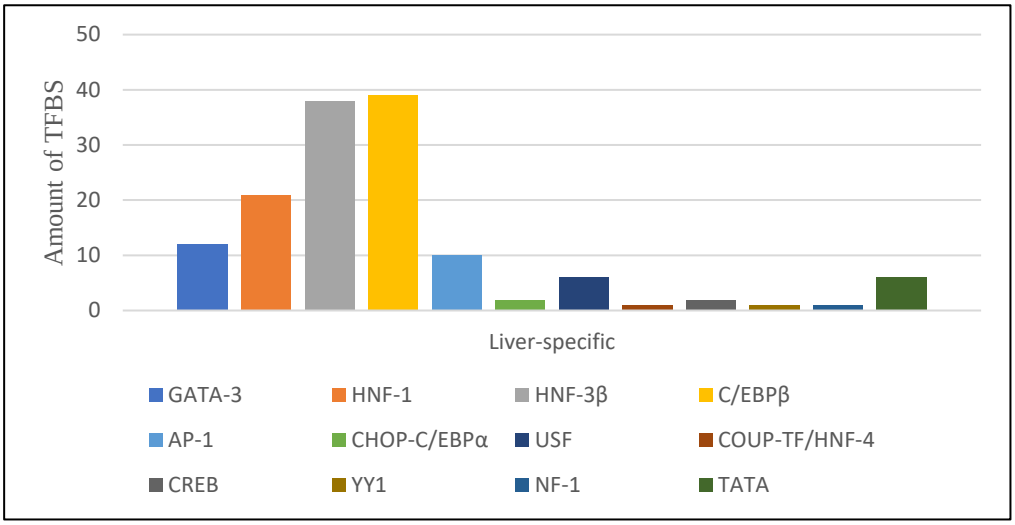

Figure 1. The predicted TFBS composition in the promoter sequence.

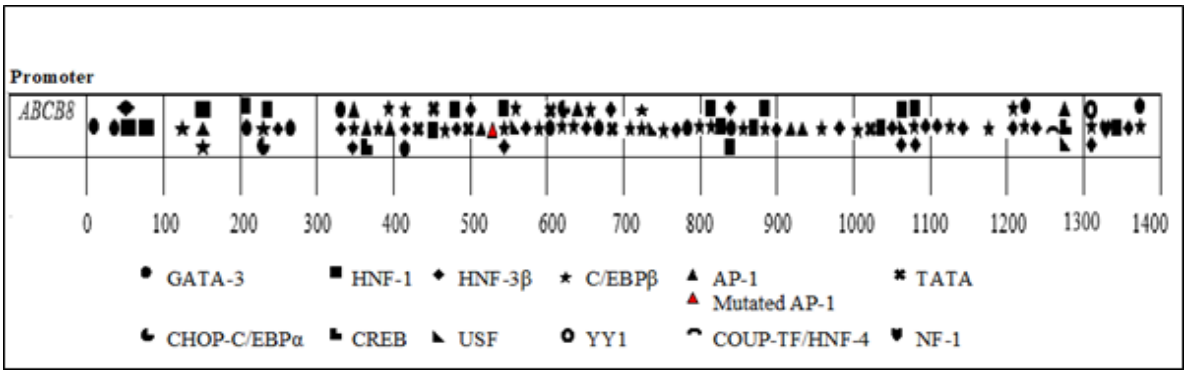

Figure 2. The TFBS distribution pattern per 100 bp interval of the *ABCB8* promoter region. The stacking TFBS represent the overlapping TFBS sites.

Mutagenic primer design

Both the mutagenic forward and reverse primers of promoter region in *ABCB8* were designed based on the region of AP-1 transcription factor. The characteristics of the mutagenic forward and reverse primers for *ABCB8* were shown in Table 1.

Table 1. The parameters of *ABCB8* mutagenic primer pair synthesis.

Primer	**Forward**	**Reverse**
Sequence (5' – 3')	TGGGGGTTTAGATATTGAAAC	AACTCGCATACATTTCAGTCATC
Length	21 bp	23 bp
Melting temperature, Tm	50.0˚C	53.2˚C
GC content	38.1%	39.1%
Oligo amount	20.3 nmoles	27.3 nmoles

Mutated *ABCB8* gene promoter analysis

After the site-directed mutagenesis, the *ABCB8* gene promoter region was cultured and sent to sequencing at First BASE to verify the identity of the gene insert. Based on Figure 3, the sequence had reached 91% similarities with the sequence from *Boechera divaricarpa* GSS (clone B25-01-F_J06). The gaps obtained were 1% (2/139). Hence, this indicated that the plasmid does not contain the promoter region of *ABCB8* gene.

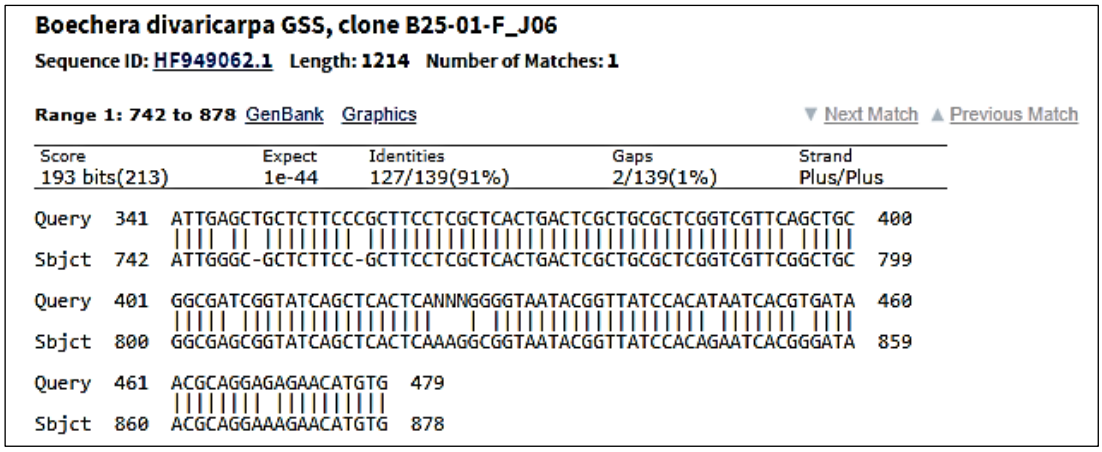

Figure 3. BLASTn analysis of the sequencing of the pGL 3.0 with mutated *ABCB8* insert.

DISCUSSION

ABCB8 is one of the subfamilies that categorized under the ABC transporters superfamily. According to Vasiliou *et al*. (2009), *ABCB8* is involved in transportation of heme, peptides and phospholipids. Besides that, *ABCB8* also is the member of the multidrug resistance (MDR) subfamily which plays a major role in chemoresistance of numerous melanoma cells (Elliott & Al-Haji, 2009). Hence, in this study, the TFBS of the *ABCB8* was analysed specifically under the liver-specific profile using MATCH (Kel *et al*., 2003). Based on the result obtained from MATCH in this study, the frequency of the TFBS found per nucleotide was 0.099785, and a total of 139 TFBSs were found in the zebrafish *ABCB8* gene promoter.

Among the TFBSs found in the promoter region of *ABCB8* gene, the AP-1 (Activator Protein 1) binding site was chosen for the mutation to take place. This is because the transcription factor AP-1 is important for the transcription regulation in *MDR1* (Daschner, Ciolino, Plouzek, & Yeh, 1999). AP-1 transcription factor which consists of dimers Jun (c-Jun, JunB and JunD) and Fos is important in the development of the liver (Trierweiler, Blum, & Hasselblatt, 2012). The c-fos also acts as a fundamental factor which controls the transcription of mRNA, protein expression of the downstream gene, cell proliferation and apoptosis (Li *et al*., 2018). The AP-1 proteins c-jun and c-fos are highly expressed in the MDR human tumours and cell lines (Daschner *et al.*, 1999). According to Trierweiler *et al.* (2012), the dimer c-Jun also functions as an oncogene in the human hepatocellular carcinoma (HCCs). In addition, the adriamycin treatment of human T-cell leukaemia had shown the activation of the c-Jun N-terminal kinase which was involved in the signalling pathway that leads to MDR (Daschner *et al.*, 1999). The AP-1 TFBS was chosen as mutation site because it is deemed as one of the most powerful contributors towards the liver function regulation by *cis*-regulatory elements (Lim *et al.*, 2019b). Mutation induced in the binding site of AP-1 leads to the conformation changes of the binding site and exerts hinge effects (Kim, Zhao, Lu, & Zhao, 2017). Besides that, mutation at the binding site also influences the binding affinity of the transcription factor to the binding site (Kim *et al*., 2017). According to Lim *et al*. (2019b), the AP-1 pairing has more influence on the liver enhancer activity as compared to the individual ones. The AP-1 site selected for mutation in this study is one of the AP-1 pair found within the zebrafish *ABCB8* promoter (Figure 2), hence it is interesting to see how the promoter activity is affected by this mutated AP-1 in future.

In this study, the TFBS that had the highest composition (39 binding sites) is the C/EBPβ or also known as nuclear factor-interleukin-6 (NF-IL6). C/EBPβ is one of the family members of CCAAT/enhancer-binding protein, which regulates the large amount genes in different functions such as acute phase response, hematopoiesis, immune function, tumour invasiveness and solid organ development (Pal *et al*., 2009). The lipopolysaccharides (LPS), interleukin-1(IL-1), interferon-gamma (IFNγ) and interleukin-6 (IL-6) are the stimuli that induced the upregulation of the C/EBPβ mRNA levels from acute phase response to inflammatory stimuli in the liver and hepatic cells (Takiguchi, 1998). Besides, complexes formed through the homodimerization and heterodimerization of C/EBPβ with other C/EBP family or with other basic leucine zippers (bZIP) protein family members able to repress and activate the transcriptional activity (Takiguchi, 1998). For example, the heteromer that formed by the complex C/EBPβ with AP-1 family member represses the transcriptional activity because the complex formed cannot bind to the C/EBPβ binding site (Takiguchi, 1998). Interestingly, the HNF-1 TFBS was also found in relatively high abundance (21 binding sites). Looking at Figure 2, the clustering of C/EBPβ and HNF-1 TFBSs occurs very frequently, especially at 400-500 bp, 800-900 bp and 1000-1100 bp regions. This phenomenon echoed the observation by Lim *et al.* (2019b) on one of the enhancers isolated. According to Lim *et al.* (2019b), this cooperative TFBS clustering strongly orchestrated the enhancer activity investigated as the deletion of this region had resulted in at least 2.7-fold drop in enhancer activity in the liver cells. Therefore, it can be postulated that the role of this C/EBPβ and HNF-1 clustering in ABCB8 zebrafish promoter may be pivotal in liver regulation and metabolism.

From the result obtained, a sum of 38 HNF-3β binding sites were found within the promoter region of the zebrafish *ABCB8* gene. According to Lau, Ng, Loo, Jasmen, and Teo (2018), HNF-3β or the forkhead box protein A2 (FOXA2), is highly expressed in the epithelial cells of the developing liver, pancreas and other tissues in the body. HNF-3β is very important during the fasted state of the organism because this transcription factor activated the transcriptional regulation of lipid metabolism and ketogenesis (Wolfrum, Asilmaz, Luca, Friedman, & Stoffel, 2004). Based on Figure 2, the HNF-3β binding sites scattered quite evenly across the *ABCB8* zebrafish promoter region. In other words, no significant HNF-3β clustering was observed. This is essential to note because Lim *et al*. (2019b) discovered that HNF-3β TFBS clustering had resulted in a drastic plunge in liver enhancer activity. The absence of HNF-3β clustering in the zebrafish *ABCB8* promoter in this study may indicate that there is no presence of any *cis*-regulatory element activity disruptor.

CONCLUSION

In this study, the sequencing result of pGL3.0 containing the promoter region of *ABCB8* gene was 100% similar to that of the *D. rerio* clone DKEYP-87A6 linkage group 24 from GenBank database. Through the TFBS analysis of the sequence specifically in liver-specific profile, the TFBSs for HNF-3β and C/EBPβ were abundantly found in the promoter region of *ABCB8*. Besides, the clustering of some TFBSs like the C/EBPβ and HNF-1, is believed to play major roles in the liver development and lipid metabolism in *D. rerio*.

Further study should focus on investigating more transcription factors in the promoter region of *ABCB8* gene. Besides that, transfection of the mutated gene into cell lines can be done to determine the regulatory activity of the transcription factor. Furthermore, the MDR mechanism of *ABCB8* in cancer cell could be unraveled by mutating the promoter region of *ABCB8*. A comprehensive review consolidating all the related researches (Lim, Chung, Hussain & Bujang, 2019c; Lim & Chung, 2020; Lau, Lim, Ishak, Abol-Munafi & Chung, 2021) would be of great benefit to those pursuing this similar line of research in future. This result may benefit for the development of new diagnostics and therapeutics for cancer and iron-associated disorder, following the promising results obtained from zebrafish knockout experiments using this promoter and its mutants in the future.

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