Auto-Regulation of the Sohlh1 Gene by the SOHLH2/ SOHLH1/SP1 Complex: Implications for Early Spermatogenesis and Oogenesis

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Abstract

Tissue-specific basic helix-loop-helix (bHLH) transcription factor proteins often play essential roles in cellular differentiation. The bHLH proteins SOHLH2 and SOHLH1 are expressed specifically in spermatogonia and oocytes and are required for early spermatogonial and oocyte differentiation. We previously reported that knocking out Sohlh2 causes defects in spermatogenesis and oogenesis similar to those in Sohlh1-null mice, and that Sohlh1 is downregulated in the gonads of Sohlh2-null mice. We also demonstrated that SOHLH2 and SOHLH1 can form a heterodimer. These observations led us to hypothesize that the SOHLH2/SOHLH1 heterodimer regulates the Sohlh1 promoter. Here, we show that SOHLH2 and SOHLH1 synergistically upregulate the Sohlh1 gene through E-boxes upstream of the Sohlh1 promoter. Interestingly, we identified an SP1-binding sequence, called a GC-box, adjacent to these E-boxes, and found that SOHLH1 could bind to SP1. Furthermore, chromatin-immunoprecipitation analysis using testes from mice on postnatal day 8 showed that SOHLH1 and SP1 bind to the Sohlh1 promoter region in vivo. Our findings suggest that an SOHLH2/SOHLH1/SP1 ternary complex autonomously and cooperatively regulates Sohlh1 gene transcription through juxtaposed E- and GC-boxes during early spermatogenesis and oogenesis.

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Introduction

Transcriptional regulation is essential for cellular differentiation. Previous studies have demonstrated that a number of transcriptional factors play important roles in early spermatogenesis and oogenesis [1,2]. Recently, several gene-knockout studies revealed that the germ cell-specific basic helix-loop-helix (bHLH) proteins SOHLH2 and SOHLH1 are expressed in spermatogonia and early oocytes [3–6] and are required for their differentiation [4–8]. The Sohlh2 transcript is upregulated shortly after birth, and the SOHLH2 protein is expressed in the adult testis by a portion of A spermatogonia throughout differentiation [6]. In mouse oogenesis, the Sohlh2 transcript is upregulated before birth [6], and its protein is expressed in primordial through primary oocytes in the ovary [3,6]. Although the Sohlh1 and Sohlh2 expression patterns are similar, the Sohlh1 transcript is upregulated following Sohlh2 expression in both early spermatogenesis and oogenesis [6]. The SOHLH1 protein is expressed by A spermatogonia throughout differentiation [3]. Since both male and female Sohlh2- and Sohlh1-null mice are infertile, and these mice have similar abnormalities in gonad histology and gene expression patterns, Sohlh2 may be upstream to Sohlh1 in the gene regulatory hierarchy. The bHLH proteins are known to form heterodimers or homodimers to bind to the consensus E-box DNA sequence CANNTG. Some bHLH proteins, such as ARNT, can transactivate target genes through homodimerization [9], while others, such as MAX-MYC, transactivate their target genes through heterodimerization [10,11]. It has been reported that SOHLH2 and SOHLH1 can form a heterodimer [6], and that the Sohlh1 mRNA levels are significantly reduced in the Sohlh2-null testis and ovary compared to the levels in wild-type gonads [6–8]. Since Sohlh1 contains several E-box (CACGTG) motifs in its promoter region (see below), it is possible that the SOHLH proteins regulate the Sohlh1 gene.

Transcription factors often function by forming complexes with other proteins. The bHLH proteins sometimes form ternary complexes with SP1, which is a zinc finger-type transcription factor [12] that binds to the consensus DNA sequence GGGCCGGGCG, called a GC-box [13]. The ternary complex binds to juxtaposed E- and GC-boxes and synergistically transactivates the adjacent promoter, as seen in Myogenin/SP1 and NeuroD1/SP1 complexes [14,15]. SP1 is widely expressed in various cell types, including spermatogonia and oocytes [16,17]. Interestingly, here we identified juxtaposed E- and GC-box sequences in the upstream region of the Sohlh1 gene of various mammalian species, and found evidence that the SOHLH proteins form a ternary complex with SP1 to regulate the Sohlh1 gene. We also identified the motifs in the Sohlh1 promoter involved...
in this regulation. These findings improve our understanding of the molecular mechanisms that regulate Sohlh1 in male and female germ-cell differentiation.

**Materials and Methods**

**Ethics statement**

Experiments involving animals were carried out in accordance with institutional guidelines under protocols (No. 21-0089) approved by the Animal Care and Use Committee of the Osaka University Graduate School of Medicine.

**Cell culture**

HEK293 cells (BioWhittaker, Walkersville, MD) were cultured in Minimum Essential Medium (Sigma-Aldrich, St. Louis, MO; Cat#M0643) supplemented with 10% heat-inactivated fetal calf serum, at 37°C.

**Western blotting and immunoprecipitation assay**

Samples were homogenized in RIPA buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.1% SDS, and 0.1% sodium deoxycholate). The extracted protein was mixed 2:1 v/v with 3x sample buffer (New England BioLabs, Beverly, MA; Cat#B7703S) and a 1/30 volume of 1.25 M diethytritol (DTT), after which it was heated at 99°C for 5 min, separated in SDS-polyacrylamide gel, and analyzed by western blotting as described previously [18].

**Reporter assays**

The promoter region of the mouse Sohlh1 gene (−1036 to −1 bp upstream of the Sohlh1 translational start site) was obtained by PCR from the genomic DNA of E14, a mouse embryonic stem cell line derived from a 129/Ola mouse strain. For E- and GC-box mutants, we used a PCR-based method using primers with mutated sequences. Promoters containing a deletion were prepared by PCR or with the appropriate restriction enzymes. These promoters were inserted into the multi-cloning sites of the pGL3-Basic vector (Promega, Madison, WI; Cat#E1751) and used for reporter assays. The pCMV-FLAG-Sohlh1, pCMV-FLAG-Sohlh2, pCAG-Sohlh1, and pCAG-Sohlh2 plasmid vectors were constructed as described previously [6]. The pcDNA3-Soohlh2-Myc and pcDNA3-Soohlh1-Myc vectors were obtained by inserting mouse Sohlh2 and Sohlh1 cDNA, respectively, into the pcDNA3-Myc-His vector (Invitrogen, Carlsbad, CA; Cat#V855-20). Mouse Sp1 cDNA obtained from testis RNA by reverse transcription followed by PCR was inserted into a pCAG-IP plasmid vector [19]. All the PCR-amplified fragments were confirmed by sequencing. The primers used in this study are available upon request.

**Reporters assays**

HEK293 cells were plated on 24-well plates at a density of 2 x 10^5 cells per well, 24 hours prior to transfection. The cells were then co-transfected with 200 ng of a reporter vector, 0.32 to 200 ng of expression vectors, and 0.1 ng of pRL-CMV normalization vector per well using HilyMax (Dojindo Molecular Technologies, Kumamoto, Japan; Cat#H357–10). After 48 hours, the total cell extracts were obtained and subjected to luciferase assays using the Dual-Luciferase Reporter Assay System (Promega).

**Chromatin-immunoprecipitation (ChIP) assay**

Testes were isolated from three wild-type mice on postnatal day (P) 8 and were fixed in 500 μl of fixation buffer (1% formaldehyde, 4.5 mM HEPES, 9 mM NaCl, 0.09 mM EDTA) for 10 min at room temperature followed by adding 55 μl of 1.5 M glycine to stop the crosslinking reaction. ChIP experiments were performed using the EZ ChIP kit (Millipore, Billerica, MA; Cat#17–371) according to the manufacturer’s instruction. After washing three times with 1 ml of ice-cold phosphate-buffered saline (PBS), testicular cells were lysed in 400 μl of SDS lysis buffer and sonicated with a sonicator (Branson, Danbury, CT). After centrifugation at 18,000 g for 5 min, 50 μl of the supernatant was diluted with 450 μl ChIP dilution buffer containing 0.5% protease inhibitor cocktail. Magna beads and rabbit anti-Sohlh1 antibody (Abcam), rabbit anti-SP1 (ChIPAb+ Sp1, Millipore; Cat#17–601) antibody, or normal rabbit IgG were added to the samples, and incubated overnight at 4°C. Then, the samples were washed once with 3 ml of Low Salt Immune Complex Wash Buffer, once with High Salt Immune Complex Wash Buffer, and twice with TE buffer. The precipitated DNA was recovered using Spin filter column, eluted in 100 μl of TE buffer, and applied to qPCR. Chromatin regions upstream of the Sohlh1 gene were amplified using specific primer pairs: 5'-TGCCCCCTGAAATTCACAGAGACG-3' and 5'-GATAGCTTGCAAGCTCTGTTTCGACG-3' for the Sohlh1 promoter region (−371 to −284); 5'-TGACACTGTCACAAACGAGGAGC-3' and 5'-ATCCAGGGTCTCCTAGCGTG-3' for a control region far upstream of the Sohlh1 promoter (−894 to −834). Accumulation of fluorescent products was monitored using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster, CA).

**Results**

**SOHLH2 and SOHLH1 form homodimers**

Previously, we demonstrated that SOHLH2 forms a heterodimer with SOHLH1 [6]. To determine whether SOHLH2 and SOHLH1 can also form homodimers, we transiently co-expressed FLAG-SOHLH2 and FLAG-SOHLH1 with SOHLH2-Myc and SOHLH1-Myc, respectively, in HEK293 cells, which endogenously express neither SOHLH2 nor SOHLH1 (Figure 1A and 1B). Western blot experiments shown in Figure 1A and 1B confirmed that the anti-FLAG antibody did not cross-react with SOHLH2-Myc or SOHLH1-Myc, and that the anti-Myc antibody did not cross-react with FLAG-SOHLH2 or FLAG-SOHLH1. Immunoprecipitation-assay bands indicating homodimerization were detected for both SOHLH2 (Figure 1C, lane 1) and SOHLH1 (Figure 1D, lane 1). These observations suggested that SOHLH2 and SOHLH1 could form homodimers in vivo, in agreement with another recent report that SOHLH2 and SOHLH1 form both heterodimers and homodimers [20].
The SOHLH2/SOHLH1 heterodimer upregulates the Sohlh1 promoter

During testicular and ovarian development, Sohlh2 mRNA is upregulated prior to Sohlh1 expression. The Sohlh1 expression in Sohlh2-null mice remains low in the testis or ovary [6]. These observations indicated that the SOHLH2 protein might regulate Sohlh1 gene activity by forming a homodimer or a heterodimer with SOHLH1. To evaluate the roles of SOHLH2 and SOHLH1 in regulating the Sohlh1 promoter activity, we introduced a luciferase reporter plasmid vector containing the 1036-bp promoter region of the mouse Sohlh1 gene, along with various amounts of plasmid vectors expressing SOHLH2 or SOHLH1, into HEK293 cells. As shown in Figure 2A, SOHLH2 or SOHLH1 alone did not markedly transactivate the Sohlh1 promoter more strongly than the reporter alone. However, introducing the reporter plasmid with 0.32 ng, 1.6 ng, 8 ng, or 40 ng each of SOHLH2 and SOHLH1 expression plasmids significantly increased the reporter gene expression 2.8-, 3.4-, 5.1-, and 7.8-fold, respectively, relative to that of the reporter plasmid alone (Figure 2A). Thus, co-expressing SOHLH2 and SOHLH1 caused a dose-dependent increase in Sohlh1 promoter activity.

Species-conserved E-boxes in the Sohlh1 promoter are important for transactivation

To determine which sequences in the Sohlh1 gene promoter are required for its transcriptional activation by SOHLH2 and SOHLH1, HEK293 cells were transfected with reporter-gene plasmids containing various lengths of the 5' upstream sequence of the Sohlh1 promoter, along with 40 ng each of SOHLH2- and SOHLH1-expression plasmid vectors. Reporter plasmids containing the 1036-bp and 321-bp upstream sequences of the Sohlh1 gene produced comparable luciferase activity (Figure 2B). However, a reporter plasmid containing the 154-bp upstream region of the Sohlh1 gene showed 70% less luciferase activity, indicating that the region from -321 to -154 of the Sohlh1 promoter contains important sequences for Sohlh1’s regulation by SOHLH2 and SOHLH1.

Since conservation between species often highlights important functional sequences, we analyzed sequences in the publicly available NCBI genomic database [http://www.ncbi.nlm.nih.gov/]. We found that the mouse Sohlh1 gene contains three E-boxes (CACGTG) from -240 bp to -284 bp upstream of its coding region, and that these sequences are well conserved in the rat. This conservation suggested that the SOHLH proteins might regulate the Sohlh1 gene through these E-boxes, which were designated E1, E2, and E3 (proximal, middle, and distal, respectively) (Figure 3).
The E-boxes are not equal in regulating the Sohlh1 gene

To determine whether the SOHLH2/SOHLH1 heterodimer regulates the Sohlh1 gene through these E-boxes, we constructed Sohlh1 promoters containing mutations in specific E-boxes (CACG TG to GGATCC) and expressed them with SOHLH2 and SOHLH1 in reporter assays. While the promoter with an E1 or E2 mutation showed approximately 25% and 50% less luciferase activity, respectively, compared with the intact promoter, the E3-mutant promoter showed approximately 75% less activity (Figure 4). A comparable reduction was seen with an E1/E2/E3 triple-mutant promoter. These observations suggested that...
the E3 box was the most important site of SOHLH2/SOHLH1 heterodimer binding.

SOHLH2, SOHLH1, and SP1 are functionally associated in Sohlh1 promoter activation

While searching for conserved sequences in the region from −321 to −154 bp upstream of the Sohlh1 gene, we also found a species-conserved GC-box (GGGGCGGGGC), which contains the binding sequence of the widely expressed transcription factor SP1, neighboring the E-boxes (Figure 3). Some bHLH proteins interact with SP1 to cooperatively activate target genes by binding to juxtaposed E- and GC-boxes [14,15]. Therefore, it was possible that the SOHLH proteins cooperate with SP1 to activate Sohlh1 through E- and GC-boxes in its promoter region.

To investigate the importance of the GC-box, we introduced the reporter vector containing the Sohlh1 promoter and expression

**Figure 3. Conserved regulatory regions of the mouse and rat Sohlh1 gene.** The underlined E- and GC-box sequences are conserved between the mouse and rat. These E- and GC-box sequences are also found in the Sohlh1 promoter region of the chimpanzee and human (not shown). doi:10.1371/journal.pone.0101681.g003

the E3 box was the most important site of SOHLH2/SOHLH1 heterodimer binding.

**Figure 4. The three Sohlh1-promoter E-boxes are not equal in regulating Sohlh1.** Reporter assays using the pGL3-Basic vector with various mutations of the E-boxes in the Sohlh1 promoter, along with pCAG-Sohlh1 and pCAG-Sohlh2 expression vectors (200 ng each). Results show the firefly/Renilla luciferase activity relative to that of the −1036 bp intact promoter, which was arbitrarily set at 1. Error bars represent the S.E.M. of the means of 3–6 separate experiments done in triplicate. P values were calculated by Student’s t-test. *P<0.05. doi:10.1371/journal.pone.0101681.g004
Figure 5. Transcriptional synergy between SP1 and the SOHLH proteins. (A) Reporter assays using the pGL3-Basic vector with the −1036 bp intact Sohlh1 promoter (200 ng), a pRL-CMV normalization vector (0.1 ng), and expression vectors (pCAG-Sohlh2 (40 ng), pCAG-Sohlh1 (40 ng), and pCAG-Sp1 (40 ng)). Results show the Sohlh1 promoter-driven firefly luciferase activity relative to that of CMV promoter-driven Renilla luciferase. The white dashed line indicates the sum of the individual transcriptional activities of SOHLH2, SOHLH1, and SP1. The black dashed line indicates the sum of the transcriptional activities of SOHLH2 + SOHLH1 and SP1. P values were calculated by Mann-Whitney test. *P<0.05. (B) Reporter assays using the pGL3-Basic vector containing the −1036 bp Sohlh1 promoter with various mutations in the E-boxes and/or GC-box (200 ng), a pRL-CMV normalization vector (0.1 ng), and expression vectors (pCAG-Sohlh2 (40 ng), pCAG-Sohlh1 (40 ng), and pCAG-Sp1 (40 ng)). Results show the firefly/Renilla luciferase activity relative to that of the intact −1036 bp Sohlh1 promoter (black bar), which was arbitrarily set at 1. P values were calculated by Welch’s t-test. *P<0.05. Error bars represent the S.E.M. of the means of 3–8 separate experiments done in triplicate. doi:10.1371/journal.pone.0101681.g005
plasmid vectors for SOHLH2, SOHLH1, or SP1, alone or in combination, into HEK293 cells. Expressing SOHLH2, SOHLH1, or SP1 alone with the wild-type Sohlh1 promoter vector increased the reporter activity by 1.1-, 1.8-, or 2.9-fold above the basal level (Figure 5A). Expressing SOHLH2 and SP1 or SOHLH1 and SP1 with the wild-type Sohlh1 promoter vector enhanced the reporter activity by 2.5-, or 4.0-fold above the basal level, respectively (Figure 5A). However, expressing SOHLH2, SOHLH1, and SP1 together increased the reporter activity by approximately 19.2-fold above the basal level, far exceeding the sum of the activation levels obtained with each factor individually (Figure 5A). Thus, SOHLH2, SOHLH1, and SP1 transactivated the Sohlh1 promoter synergistically.

SOHLH1 binds to SP1

To determine whether SOHLH proteins interact physically with SP1, we co-expressed SP1 and FLAG-tagged SOHLH1 or SOHLH2 in HEK293 cells. The FLAG-tagged SOHLH proteins immunoprecipitated from cell lysates with an anti-FLAG antibody were then analyzed by western blotting with an anti-SOHLH antibody. These experiments showed that FLAG-SOHLH1 could associate with co-expressed SP1 (Figure 6A). On the other hand, the co-expression of FLAG-SOHLH2 and SP1 did not reveal any detectable association between SOHLH2 and SP1 (Figure 6B).

Considering the heterodimerization of SOHLH2 and SOHLH1, SOHLH1 might act as a bridge between SOHLH2 and SP1, resulting in the formation of the SOHLH2/SOHLH1/SP1 complex. As it was reported that the DNA-binding domain of SP1 and the HLH domain of MYOGENIN or NEUROD1 mediate protein-protein interactions [14,15], it is possible that the observations suggested that the SOHLH proteins, in cooperation with SP1, transactivate Sohlh1 through juxtaposed E- and GC-boxes.
HLH domain of SOHLH1 also associates with SP1’s DNA-binding domain.

SOHLH1 and SP1 are recruited to the Sohlh1 promoter region in vivo

To examine whether SOHLH1 and SP1 are recruited to the Sohlh1 promoter region in vivo, we performed ChIP assay using P8 testes (Figure 7). By qPCR, the Sohlh1 promoter region (−371 to −284) was shown to be significantly enriched in SOHLH1- and SP1-immunoprecipitated chromatin fractions, while a control region far upstream of the Sohlh1 promoter (−8946 to −8834) was not. These results indicated that the sequences located immediately upstream of the Sohlh1 transcription start site are bound by SOHLH1 and SP-1 in vivo.

**Discussion**

In our previous study, we reported that abnormalities in the testes and ovaries of Sohlh2-null mice are similar to those seen in Sohlh1-null mice, and that Sohlh1 transcription is downregulated in the gonads of Sohlh2-null mice [6]. We also demonstrated that SOHLH2 can form a heterodimer with SOHLH1 [6]. In the current study, we showed that SOHLH2 and SOHLH1 could also form homodimers (Figure 1C, D). We further demonstrated that SOHLH2 and SOHLH1, expressed together, upregulated the Sohlh1 promoter through its E-boxes, while SOHLH2 or SOHLH1 alone upregulated this promoter activity only weakly (Figure 2A).

Similar observations have been made for MAX and MYC. MAX, a MYC-family bHLH protein, not only forms homodimers, but also forms heterodimers, preferentially with MYC [21,22]. The MAX/MYC heterodimer binds to the E-box sequence CACGTG with higher affinity than does MAX or MYC alone [21]. MAX homodimers have no transcriptional activity, but the MAX/MYC heterodimer is a principal transcriptional activator [10,11]. In this respect, SOHLH2 and SOHLH1 appear to function similarly to MAX and MYC.

We next demonstrated that the SOHLH proteins and SP1, which are all present in germ cells, are functionally linked. A species-conserved GC-box, which contains the SP1 consensus binding sequence, is adjacent to the Sohlh1 promoter’s E-boxes (Figure 3). Some bHLH proteins, including NEUROD1, interact with SP1 to synergistically activate target genes containing juxtaposed E- and GC-boxes [14,15,23]. It has been reported that the synergy between E12/NEUROD1 and SP1 occurs when the bHLH proteins recruit SP1 and stabilize its DNA-binding [15]. The association we observed between SOHLH1 and SP1 suggests that the SOHLH2/SOHLH1 heterodimer might also recruit SP1 and stabilize SP1’s binding to the GC-box, thereby synergistically activating the Sohlh1 promoter activity to its greatest extent (Figure 8). Consistent with this notion, ChIP assay using P8 testes showed that SOHLH1 and SP1 bind to the Sohlh1 promoter region in vivo (Figure 7).

Of the three E-boxes (E1, E2, and E3), the reporter activity was reduced most greatly by a mutation in E3 (Figure 4). The ternary complex of NEUROD1, E12, and SP1 requires proper spacing between the E- and GC-boxes for the strongest promoter activation [15]. The SOHLH2/SOHLH1/SP1 complex also requires a particular spacing between the E- and GC-boxes for maximum promoter activation, with the E3 E-box being at the most appropriate distance from the GC-box for promoter activation by the SOHLH2/SOHLH1/SP1 ternary complex.

In early spermatogenesis and oogenesis, the Sohlh2 gene is upregulated prior to the Sohlh1 gene [6]. However, SOHLH2 was barely able to transactivate the Sohlh1 promoter in the absence of SOHLH1 (Figure 2A), so Sohlh2 upregulation alone may not lead to Sohlh1’s transcription in vivo. The mechanism that initially activates Sohlh1’s transcription remains unknown. It is possible that SOHLH2 homodimers have weak transcriptional activity. Similarly, although MAX homodimers are generally thought to repress transcription, they have been found to activate transcription at low levels in a yeast system [24]. Another possibility is that other factors are responsible for turning on Sohlh1’s transcription (Figure 8A). Since Sohlh1 transcription is detected in the gonads of Sohlh2-null mice [6], the Sp1 transcript was consistently observed throughout spermatogenesis and oogenesis (data not shown), and
ternary complex might be a key factor in this transcriptional
SOHLH2/SOHLH1/SP1 complex might directly regulate ex-
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directed transcription (Fig 8A). In Sohlh2-null mice, KIT expression is downregulated in both the
tests and ovary, and KIT-positive germ-cell differentiation is disturbed [6]. In Sohlh2-null mice as well, KIT-positive germ-cell differentiation appears to be disturbed in the tests and ovary [3,8]. KIT is expressed in Aγδ spermatogonia and in primordial-to-
growing oocytes, corresponding to the SOHLH2 and SOHLH1 expression. SP1 is also expressed in spermatogonia [16] and oocytes [17]. Recently, Barrios et al. [25] reported that SOHLH2 and SOHLH1 control the KIT expression during postnatal male germ-cell development. The KIT proximal promoter is reported to contain E- and GC-boxes [26,27]. These reports suggest that the

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Acknowledgments

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Author Contributions

Conceived and designed the experiments: ST JiM. Performed the experiments: ST TY JM. Analyzed the data: ST TY JM. Wrote the paper: ST TY JM.

Sohlh1 Auto-Regulation by SOHLH2/SOHLH1/SP1

SP1 alone was able to activate weak but detectable Sohlh1

expression is off targeted by the E- and GC-boxes of the

expressed in male germ-cell development. The SP1 complex might directly regulate

and acetylated histones in the retinoic acid-dependent activation of guanylyl

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114: 499–509.

113: 637–648.

112: 1239–1249.

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109: 1593–1603.

108: 593–603.

107: 325–337.

106: 2919–2927.

105: 1961–1971.

104: 2299–2307.

103: 7118–7124.

102: 3393–3402.

101: 1167–1172.

100: 2327–2331.

99: 1627–1630.

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97: 1007–1010.

96: 3773–3783.

95: 1217–1224.

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