Estradiol-Estrogen Receptor α Mediates the Expression of the CXXC5 Gene through the Estrogen Response Element-Dependent Signaling Pathway

**Pelin Yaşar, Gamze Ayaz and Mesut Muyan**

SUPPLEMENTAL DATA

Fig. 1. Assessment of the endogenous CXXC5 protein in MCF7 cells. (**a**) Overexpressed CXXC5 protein overshadows the endogenous CXXC5. MCF7 cells were transfected with pcDNA3.1(-) bearing none (Vector, V) or the Flag-CXXC5 (F-C5) cDNA. Twenty-four hour after transfections, cells were subjected to protein extraction. Equal amounts (100 μg) of nuclear extracts of the parent vector (V) or Flag-CXXC5 cDNA transfected cells were run onto SDS 10%-PAGE and subjected to WB using a CXXC5-specific antibody, ab106533. Star in the vector transfected (V) lane denotes the putative endogenous CXXC5 protein, while arrows indicate the overexpressed Flag-CXXC protein in corresponding lane. Molecular weight marker is shown in kDa. A representative image from two independent experiments is shown. (**b & c & d**) MCF7 cells were transfected as indicated. Twenty-four hour after transfection cells were lysed and separated into nuclear and cytoplasmic fractions. To prevent the overshadowing effect of the overexpressed WT-CXXC5 or Flag-CXXC5 protein at 100 μg, 25 ug protein from the nuclear (**b**) and cytoplasmic (**c**) extract of the overexpressed WT-CXXC5 or Flag-CXXC5 protein as well as 100 μg of protein extracts from Vector (V) transfected cells were subjected to WB using ab106533. Star denotes the endogenous CXXC5 protein, while arrows indicate the overexpressed CXXC proteins. Molecular weight marker is shown in kDa. A representative image from two independent experiments is shown. (**d**) Overexposed image, six second exposure, of the WB image shown in B, which was exposed for two seconds. In all panels, β-Actin and HDAC1 were used as loading controls for cytoplasmic and nuclear extracts; the presence, albeit low amounts, of β-Actin in the nuclear extract and HDAC1 in the cytoplasmic extract indicates somewhat cross-contamination of cellular fractions. This suggests also that the detection of CXXC5 proteins in the cytoplasmic extracts is likely due to the contaminating nuclear extract.

Fig. 2. Nucleotide sequences of the estrogen responsive region of CXXC5. The 305 base-pair long *CXXC5* locus containing an ERE sequence was obtained by PCR using the genomic DNA of MCF7 cell and sequenced. The sequence (query) was then compared to sequences in human genome using Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The ERE sequence (underlined in red) resides between residues -242 through -255 from the first encoding ATG (A being +1) codon of *CXXC5,* underlined in black. The PCR generated DNA fragment was inserted into the pGL3-Basic vector for the construction of the pGL3-CXXC5 reporter plasmid.

Fig. 3. Electrophoretic Mobility Shift Assay (EMSA). (**a**) Cell extracts (CE; 10 μg) of MDAMB231 cells transfected with pcDNA3.1(-) bearing none (Vector, V) or the Flag-ERα cDNA were subjected to EMSA using biotinylated DNA (40 fmol) with (+) or without (-) the Flag-M2 antibody (Flag-M2) in the absence (-) or presence (+) of cold competitor at indicated amounts. ERα-ERE denotes the protein-bound biotinylated ERE. ERE indicates the unbound (free) biotinylated ERE. (**b**) MDAMB231 cells were transfected with pcDNA3.1(-) bearing none (V) or the Flag-ERα cDNA. Cell extracts were subjected to EMSA using biotinylated CXXC5-ERE (40 fmol). Specific binding of ERα to CXXC5-ERE was examined in the presence (+) of excess (2500 fold) cold (unlabeled) DNA fragment with no-ERE features (5'-CTGTCAGTATCAGTTACAGGTCTA-3', upper stand).

Table 1. Nucleotide sequences of siRNAs. Shown are the nucleotide sequences of siRNAs used in experiments to assess the identity of the endogenous CXXC5 protein.

Supplemental Data, Figure 1
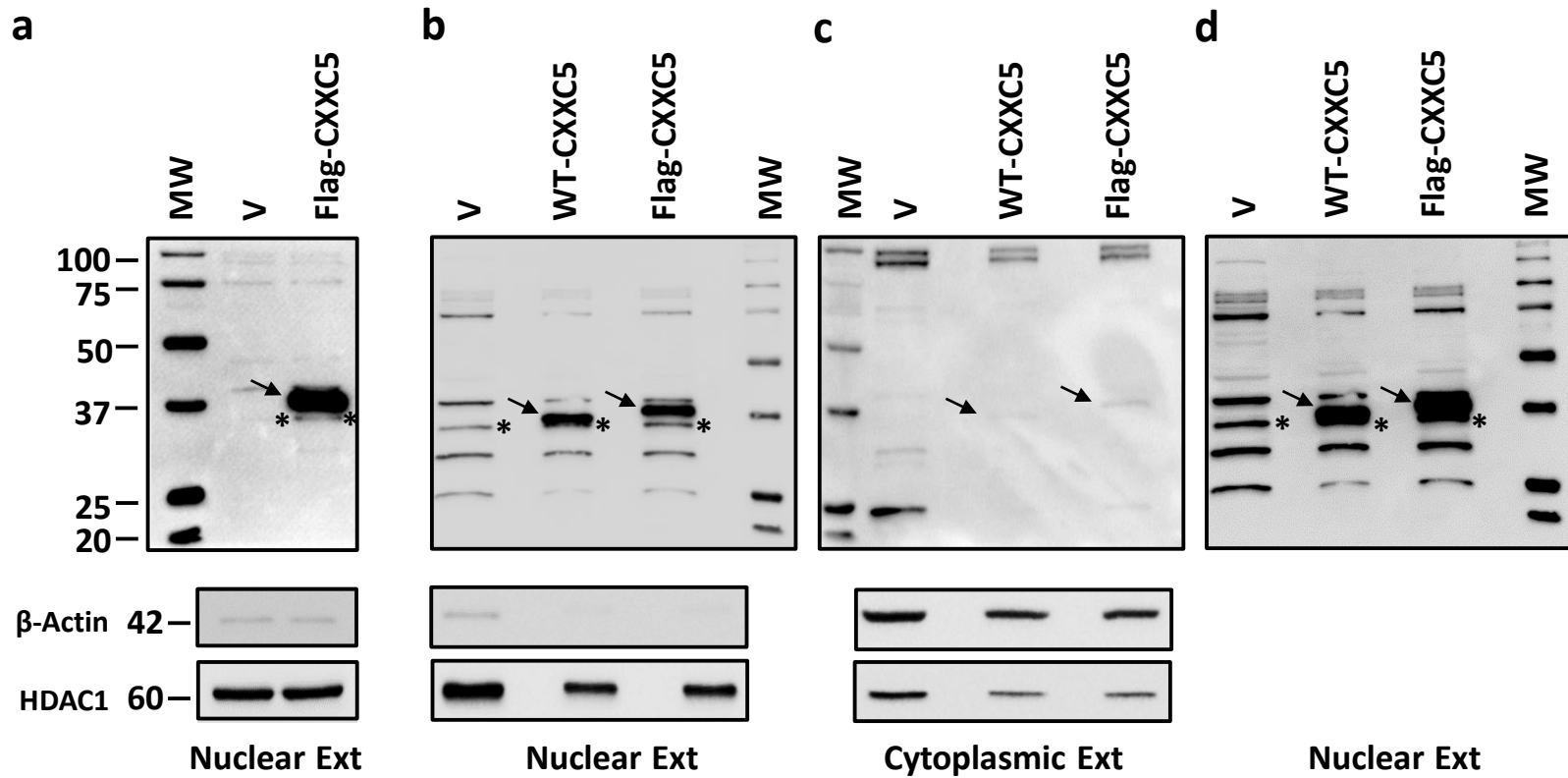
Supplemental Data, Figure 2

Homo sapiens chromosome 5, alternate assembly CHM1_1.1
Sequence ID: ref|NC_018916.2| Length: 180347728 Number of Matches: 1

Range 1: 138492850 to 138493157 GenBank Graphics   ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
569 bits(308)	5e-160	308/308(100%)	0/308(0%)	Plus/Plus

Features: CXXC-type zinc finger protein 5
         CXXC-type zinc finger protein 5

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                                                            -255
Query 1   CTCACCAGGATGGATCTGGTGCTTAATAAATGCCTGGTCAAGCACATGGTGGTCAGGATG 60
                  |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 138492850 CTCACCAGGATGGATCTGGTGCTTAATAAATGCCTGGTCAAGCACATGGTGGTCAGGATG 138492909
                  -242
Query 61   ACAGGACCGTTGATAGTGGCGGTGGTGGCGATGTTGAAGGGGGAGGTGTTCACTGCTGCC 120
                  |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 138492910 ACAGGACCGTTGATAGTGGCGGTGGTGGCGATGTTGAAGGGGGAGGTGTTCACTGCTGCC 138492969

Query 121   CTGACCCTGTATCCTCTTGTGACAGAGTGAAGACATTTCCACCTGGACACCTGACCATGT 180
                  |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 138492970 CTGACCCTGTATCCTCTTGTGACAGAGTGAAGACATTTCCACCTGGACACCTGACCATGT 138493029

Query 181   GCCTGCCCTGAGCAGCGAGGCCCACCAGGCATCTCTGTTGTGGGCAGCAGGGCCAGGTCC 240
                  |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 138493030 GCCTGCCCTGAGCAGCGAGGCCCACCAGGCATCTCTGTTGTGGGCAGCAGGGCCAGGTCC 138493089

Query 241   TGGTCTGTGGACCCTCGGCAGTTGGCAGGCTCCCTCTGCAGTGGGGTCTGGGCCTCGGCC 300
                  |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 138493090 TGGTCTGTGGACCCTCGGCAGTTGGCAGGCTCCCTCTGCAGTGGGGTCTGGGCCTCGGCC 138493149
                  +1
Query 301   CCACCATG 308
                  ||||||||
Sbjct 138493150 CCACCATG 138493157
```

From: http://blast.ncbi.nlm.nih.gov/Blast.cgi

**a**

| Con-ERE | + | + | + | + | + | + | + | + |
|---|---|---|---|---|---|---|---|---|
| CE | − | + | + | + | + | + | + | + |
| Flag-M2 Ab | − | − | + | − | − | − | − | − |
| Cold Con-ERE | − | − | − | 250x | − | − | − | − |
| Cold CXXC5-ERE | − | − | − | − | 2.5x | 25x | 250x | 2500x |

ERα-ERE

ERE—
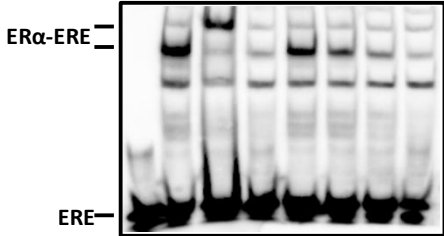
**b**

| CXXC5-ERE | + | + | + |
|---|---|---|---|
| Vector | + | − | − |
| ERα | − | + | + |
| Cold Non-ERE | − | − | 2500x |
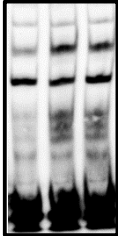
**Supplemental Data, Table 1**

| siRNAs | siRNA sequence (5' to 3') | Target |
|---|---|---|
| Hs_CXXC5_2 | CAGCAGTTGTAGGAATCGAAA | In the ORF of CXXC5 |
| Hs_CXXC5_7 | CAAGAGCGGTATCATCAGTGA | In the ORF of CXXC5 |
| Hs_CXXC5_9 | AGGGATTCGGGCGAAGACAAA | In the 3'UTR of CXXC5 |
| Hs_CXXC5_10 | TCAGATTTGCAAATTCAGAAA | In the ORF of CXXC5 |
| AllStars Negative Control | Proprietary sequence | |