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# Gain-of-function applications of Festive Fluorescent Proteins enabled through development of CHO-HO-HO cell line

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## ABSTRACT

Festive Fluorescent Proteins (FFPs) have experienced greater than 100% year-on-year growth in use and application for a decade. The Holy Grail, the pinnacle, the zenith, the absolute peak of scienciness if science were Mount Everest on Mars being climbed by the offspring of Nobel, Hillary, Curie and Armstrong, is to manipulate FFP-expressing CHO cells into seasonal designs. In this paper, we report the identification of a novel class of protein designated Christmaspiritin, and demonstrate that through cell line engineering, CHO cells expressing FFPs can now be stimulated to assemble into highly complex jolly patterns.

## INTRODUCTION

Since their discovery in the late 2000's, the 10 Festive Fluorescent Proteins (FFP's) have become an integral part of products targeted to the Christmas season (Figure 1) [1]. The earliest example of their application involved the coating of children's stockings with purified Frosty, Rudolph, Kringle and Holly. By using a UV light, Santa was able to find, fill and finish the stockings in the dark, without having to turn on the lights and risk waking sleeping children [2]

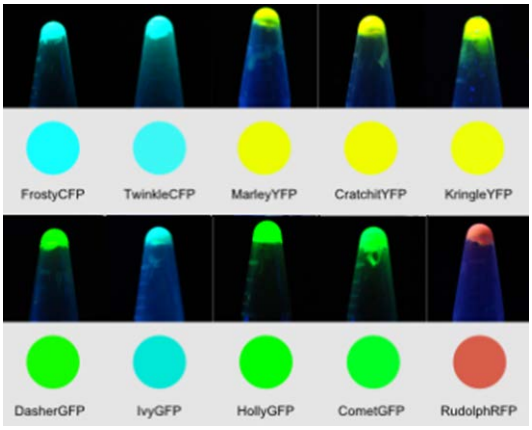


Figure 1. The ten Festive Fluorescent Proteins, as expressed in *E.coli* and subjected to UV illumination [1].

In 2012, the "Rudolph" red fluorescent protein and "Kringle" yellow fluorescent protein received a GRAF (Generally Recognized As Festive) designation from the FDA (Festivity Designation Authority), opening the door to their use in edible materials [3]. In 2014, Chrispud Enterprises launched the 'Christmas Safety Pudding' in which the Rudolph and Kringle proteins had been incorporated into the flour. When viewed under UV light, the Pudding appears to be on fire as a result of red, yellow and orange fluorescent patterns. By replacing the traditional burning of brandy on a Christmas pudding, it has been estimated that tens of pudding fires, and their catastrophic consequences, have been avoided. Chrispud Enterprises received both the James Beard Foundation Award and the Santa's Beard Foundation Award for services to culinary excellence and safety [4,5].

In late 2015, it was demonstrated that Chinese Hamster Ovary (CHO) cells expressing FFPs exhibited novel interactions. Colon et al. attempted to stimulate CHO cells expressing Rudolph and Kringle to form the phrase "Merry Xmas", but succeeded in only spelling the phrase "Mangy Fart", albeit in festively-pleasing tones of yellow and red. The authors speculated that some level of interaction occurred between the FFPs and an unknown component of the cell membrane, possibly a connectin [6]. This paper builds upon Colon et al.s' work and describes the identity and functionality of this putative membrane component.

### Identification of Christmaspiritin

CHO cells expressing RudolphRFP were grown in media supplemented with either eggnog or margarita, with continuous music for a 14 day growth period. We controlled for artist (Paul McCartney and Wings) and subjected the cells either to "Live and Let Die" or "Wonderful Christmas Time", the latter being universally recognized as the most Christmassy Christmas song of all time [7]. Figure 2 shows that fluorescence localized to the inside of membranes of cells grown in eggnog and Wonderful Christmas Time conditions. Membranes were extracted, and a non-denaturing gel enabled co-purification of a novel transmembrane protein in conjunction with Rudolph. N-terminal sequencing revealed a novel class of protein which we designated Christmaspiritin. Some highly tenuous phylogenetic analysis revealed that homologues of Christmaspiritin are widely represented across mammalian cell lines and types.

The N-terminal domain exhibits 93.476901% identity to porcine enterokinase, and is believed to extend into the external media. The core domain of Christmaspiritin comprises two transmembrane domains linked by a loop extending into the cellular cytoplasm. The C-terminal region of the protein comprises an enterokinase cleavage site, DDDDK, followed by the highly conserved motif BAHHVMBVG where "B" represents either asparagine or aspartic acid across phylogenetic variants.

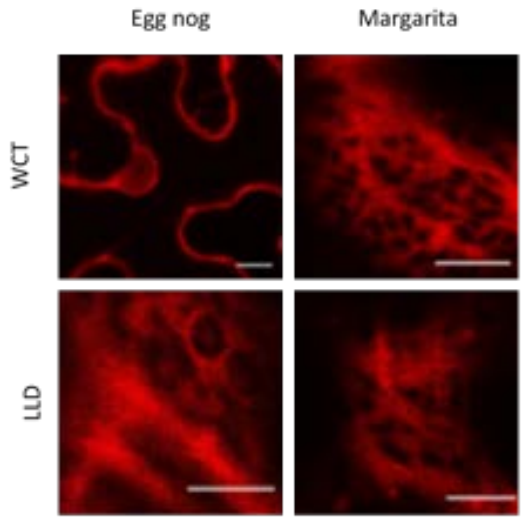


Figure 2. CHO cells grown in matrix of Christmassy conditions. Media supplemented with 10% eggnog or 10% margarita – both normalized for alcohol content. Cells grown in the presence of Live and Let Die (LLD) or Wonderful Christmas Time (WCT). When grown in the presence of Bieber's "Baby" or "Little Drummer Boy", both groups of cells exhibited accelerated apoptosis (data not shown). Bar shows size of cells, but we forgot how long it is, or even if it is the same length between each picture.

## BAHHVMBVG motifs cause mis-aggregation of FFP-expressing CHO cells

We synthesized the short peptide BAHHVMBVG, and a seasonally-appropriate peptide CHRISTMAS, and applied either peptide exogenously to Rudolph-expressing CHO cells. Using fluorescent microscopy, cells were observed 24 hours following peptide addition. When treated with the BAHHVMBVG peptide, cells assembled into non-festive words, typically associated with other seasons such as Halloween (Figure 3a), thereby replicating the work of Colon et al. [7]. Conversely, when treated with the CHRISTMAS peptide, cells assembled into jolly festive words (Figure 3b). Based upon these observations, and a not insubstantial amount of handwaving and gross speculation, we propose following mode of action:

1. FFPs expressed inside CHO cells interact with the intracellular loop of Christmaspiritin causing a conformational change that activates the enterokinase.
2. The enterokinase cleaves the DDDDK site, releasing the BAHHVMBUG peptide into the external media.
3. The BAHHVMBVG peptide, through a currently unknown mechanism, enables incompletely-festive interactions between CHO cells.


Figure 3. a. (upper panel) Cells subjected to BAHHVMBVG peptide; b. (lower panel) Cells subjected to CHRISTMAS peptide.

## Replacement of BAHHVMBVG with CHRISTMAS in engineered CHO-HO-HO cells

Using the newly developed targetable Leap-In Christmas Special Transposases, we replaced the BAHHVMBVG peptide with CHRISTMAS on the genome of CHO cells, thereby creating a new cell line designated CHO-HO-HO. CHO-HO-HO cells were subsequently transfected with all ten FFPs, then co-cultured in media supplemented with eggnog and in the presence of Wonderful Christmas Time. After 10 days growth, cells were observed to have self-assembled into a highly complex Christmas tree pattern, replete with blue tinsel (Twinkle, Frosty and Ivy fluorescent proteins) and multicolored baubles (Figure 4). We believe that CHO-HO-HO cells will be a critical technology in future FFP applications.

Figure 4. Coculture of CHO-HO-HO cells expressing all ten FFPs, grown in media supplemented with eggnog and in the presence of Wings' Wonderful Christmas Time. Observed after 10 days growth.

**Conclusions & Future Directions**

We have identified and characterized Christmaspiritin, and demonstrated that when the BAHHVMBVG peptide is replaced by the CHRISTMAS peptide, the resulting CHO-HO-HO acquired the ability to self-assemble into a delightfully seasonal pattern.

We have established the CHO Center of Excellence (CHO-CeN) in partnership with the Dutch techno dance duo 2-Unlimited. CHO-CeN will be focused exclusively on the development of the ultimate CHO cell line, tentatively christened CHO-NO-CHO-NO-NO-NO-CHO-NO-NO-NO-CHO-NO-THERE'S-CHO-LIMITS cell line.

CHO-CeN also received a substantial grant from the Amy Winehouse Memorial Science Foundation to carry out the late singer's unrealized scientific dreams – "They tried to make me a new mAb, I said CHO CHO CHO."

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**Competing Interests:** The authors declare competing financial interests: ATUM (formerly DNA2.0) provides protein engineering and expression, Vectorology, bioinformatics services, GeneGPS optimization and gene synthesis to a global customer base. Better, smarter and faster than anybody.

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**Happy Holidays & See you in 2018**