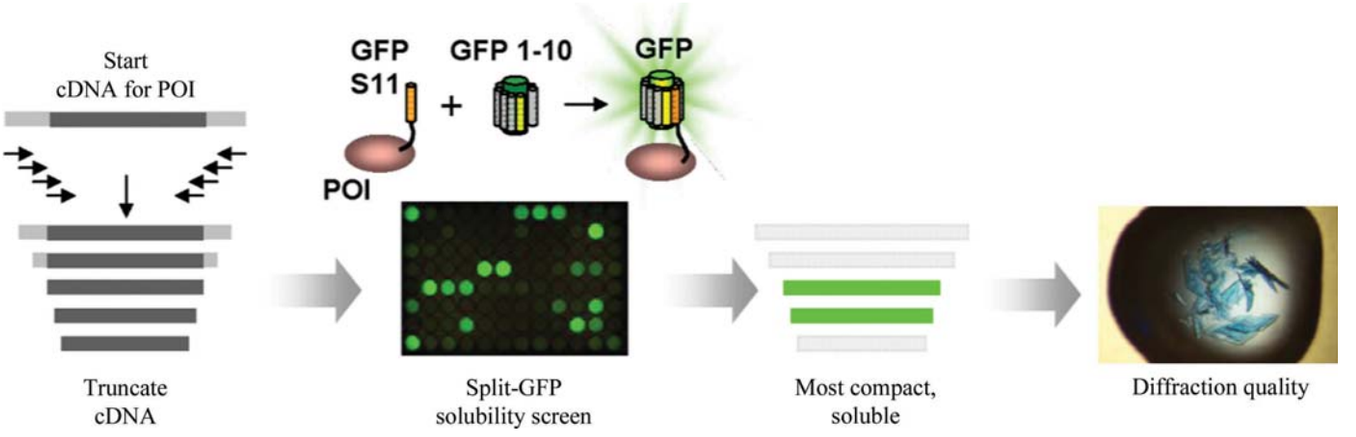

Figure 4.3.4.1
A domain-trapping strategy to engineer soluble variants of a protein of interest (POI) for crystallization using the split-GFP complementation methodology. (Figure courtesy of Dr Geoff Waldo, LANL.)

multiple free cysteines. In She2p, an RNA-binding protein, four cysteines (Cys14, Cys68, Cys106 and Cys180) were mutated to serines in order to overcome oxidation and aggregation (Niessing *et al.*, 2004). In an extreme case, that of human maspin, which is a serpin with antitumour activities, all unpaired cysteines were mutated (C20S, C34A, C183S, C205S, C214S, C297S, C373S) in an effort to obtain a soluble crystallizable variant (Al-Ayyoubi *et al.*, 2004).

4.3.4. Optimization of target constructs

The N- and C-termini of proteins are often flexible and unstructured (Thornton & Sibanda, 1983), creating a potential entropic impediment to crystallization. Initially, the preferred way to circumvent this problem was to use limited proteolysis to trim off the ends, leaving the stable core of the target protein. This strategy remains useful, particularly in its *in situ* version, in which trace amounts of proteases are added directly to crystallization screens (Dong *et al.*, 2007; Wernimont & Edwards, 2009). However, on the downside it introduces the possibility of heterogeneity in the sample owing to incomplete proteolysis. An alternative route is to first identify the smallest functional fragment of the target protein and to then design and overexpress an appropriately modified gene. A number of options are possible. The simplest is the direct prediction of intrinsically disordered regions from the amino-acid sequence alone (Obradovic *et al.*, 2003; He *et al.*, 2009). The functional core units can also be identified experimentally by mass spectrometry following limited proteolysis (Cohen *et al.*, 1995). Alternatively, deuterium–hydrogen exchange coupled to mass spectrometry (DXMS) may be used to identify fast-exchanging amides that map to unstructured fragments (Hamuro *et al.*, 2003; Pantazatos *et al.*, 2004; Sharma *et al.*, 2009).

Importantly, the choice of optimal N- and C-termini may also critically influence the solubility of the target protein. For example, in the case of MAPKAP kinase 2, 16 truncation variants were assayed, all of which contained the catalytic domain, and were shown to have dramatically differing solubilities and propensities for crystallization (Malawski *et al.*, 2006). Similarly, a series of truncations were screened in order to identify a soluble and crystallizable variant of a three-domain fragment of the Vav1 guanine nucleotide-exchange factor (Brooun *et al.*, 2007). In both these cases only a limited number of rationally designed constructs were screened. However, to increase the prospects of success it is also possible to utilize much larger libraries of variants and screen them *in vivo* using the high-throughput split-GFP complementation assay (Fig. 4.3.4.1; Cabantous & Waldo, 2006).

Another troublesome problem associated with flexible termini is their occasional propensity to form multiple intermolecular contacts, leading to crystal forms that contain multiple copies of the target protein in the asymmetric unit. This has been observed, for example, for *Plasmodium falciparum* peptide deformylase, in which removal of three residues from the N-terminus reduced the number of subunits in the asymmetric unit from ten to two (Robien *et al.*, 2004).

In addition to the disordered N- and C-termini, target proteins may contain internal unstructured regions such as subdomains or loops which can be removed or shortened to reduce conformational heterogeneity. For example, the construct used in the successful crystallization of the HIV gp120 envelope glycoprotein had two flexible loops which were replaced with Gly-Ala-Gly linkages to obtain a crystallizable variant (Kwong *et al.*, 1998, 1999). In the case of 8*R*-lipoxygenase the replacement of a flexible Ca^{2+}-dependent membrane-insertion loop consisting of five amino acids by a Gly-Ser dipeptide resulted in crystals that diffracted to a resolution 1 Å higher than the wild-type protein (Neau *et al.*, 2007). An interesting variation of this approach was introduced for the preparation of crystals of the β-subunit of the signal recognition particle receptor. A 26-residue flexible loop was removed, but instead of replacing it with a shorter sequence the authors connected the native N- and C-termini of the protein using a heptapeptide GGGSGGG, thus creating a circular permutation of the polypeptide chain (Schwartz *et al.*, 2004).

Given that the majority of eukaryotic proteins contain at least one stretch of 40 or more disordered residues (Vucetic *et al.*, 2003), optimization of crystallization targets by removal of these sequences is likely to become a routine strategy.

4.3.5. The use of fusion proteins for crystallization

Tags are routinely used in heterologous protein expression in order to enhance folding and solubility and to facilitate purification (Uhlen *et al.*, 1992; Malhotra, 2009). They are either short oligopeptides, such as a hexahistidine, with unique affinity properties or well expressed and highly soluble proteins, such as GST (glutathione *S*-transferase), MBP (maltose-binding protein) or thioredoxin. The tags are inserted into the expression vectors