

# Organic & Biomolecular Chemistry

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

*Accepted Manuscripts* are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the [Information for Authors](#).

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard [Terms & Conditions](#) and the [Ethical guidelines](#) still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.



## Organic &amp; Biomolecular Chemistry

## EDGE ARTICLE

## Fluorescent IGF-II analogues for FRET-based investigations into the binding of IGF-II to the IGF-1R

Received 00th January 20xx,  
Accepted 00th January 20xx

J. M. Cottam Jones,<sup>a</sup> P. W. R. Harris,<sup>c,d,e</sup> D. B. Scanlon,<sup>a</sup> B. E. Forbes,<sup>f,g</sup> M. A. Brimble<sup>c,d,e</sup> and A. D. Abell<sup>a,b</sup>

DOI: 10.1039/x0xx00000x

www.rsc.org/

The interaction of IGF-II with the insulin receptor (IR) and type 1 insulin-like growth factor receptor (IGF-1R) has recently been identified as potential therapeutic target for the treatment of cancer. Understanding the interactions of IGF-II with these receptors is required for the development of potential anticancer therapeutics. This work describes an efficient convergent synthesis of native IGF-II and two non-native IGF-II analogues with coumarin fluorescent probes incorporated at residues 19 and 28. These fluorescent analogues bind with nanomolar affinities to the IGF-1R and are suitable for use in fluorescence resonance energy transfer (FRET) studies. From these studies the F19Cou IGF-II and F28Cou IGF-II proteins were identified as good probes for investigating the binding interactions of IGF-II with the IGF-1R and its other high affinity binding partners.

### Introduction

Insulin-like growth factor II (IGF-II) is a 67-residue regulatory peptide that binds with high affinity to three receptors; the insulin receptor (IR), type 1 insulin-like growth factor receptor (IGF-1R), and type 2 insulin-like growth factor receptor (IGF-2R). The binding of IGF-II to these receptors promotes a range of responses, including cell growth, proliferation, differentiation and apoptosis.<sup>1-6</sup>

Deregulation of the IGF system results in elevated levels of circulating IGF-II with an associated increase in binding to the IGF-1R and IR-A (insulin receptor isoform A).<sup>6-15</sup> Activation of both receptors can then promote cancer cell growth and metastasis.<sup>16, 17</sup> While a structure of insulin bound to a fragment of the IR has been reported,<sup>18, 19</sup> detailed structural information on the interaction of IGF-II with either the IR-A or IGF-1R remains elusive.

A comparison of IGF-II and IGF-II analogues with the related

ligands, IGF-I and insulin in competition binding assays on soluble receptors or cells expressing either the IR-A or the IGF-1R, does however, provide some information on the mechanisms of interaction of IGF-II with IGF-1R and IR-A.<sup>19-32</sup>

Alanine mutagenesis of IGF-II has identified two separate binding sites (defined as sites 1 and 2) that are critical for binding to the IGF-1R and IR and are analogous to the two insulin binding sites that bind the IR.<sup>33</sup> However, precise molecular details of the contact residues within the receptors remain ill-defined. X-ray crystallographic analysis of insulin bound to a fragment of the IR<sup>18, 19</sup> suggests that the site 1 residues of IGF-II most likely contact the leucine-rich domain 1 and  $\alpha$ -CT peptide.

Here we present the convergent synthesis of F19Cou IGF-II and F28Cou IGF-II and their use in a fluorescence resonance energy transfer (FRET) based study to define the interactions of IGF-II with the IGF-1R. A coumaryl glycine acceptor (see Scheme 1) was incorporated into IGF-II at Phe<sup>28</sup> and at Phe<sup>19</sup> to probe interactions with sites 1 and 2, respectively. The coumaryl glycine probe has advantages of a strong quantum yield and a large Stokes shift, while also exhibiting appropriate spectral overlap with tryptophan (Trp) as a FRET donor.<sup>34-41</sup> Trp was deemed a suitable donor as these residues were identified as being positioned nearby likely IGF-II binding sites through analysis of the X-ray crystal structure of the Insulin:IR complex as well as IR and IGF-1R mutagenesis data.<sup>18, 42, 43</sup>

There are three reports on the chemical synthesis of IGF-II and all utilise a similar linear approach that requires multi-step post-cleavage deprotections and lengthy multi-step purification protocols.<sup>44-47</sup> Furthermore, none are suitable for the incorporation of unnatural amino acids. The yield of IGF-II is presented in only two of these syntheses and this is somewhat problematically reported to be of the order of 2%

<sup>a</sup> Department of Chemistry, The University of Adelaide, Adelaide 5001, SA, Australia.

<sup>b</sup> ARC Centre of Excellence for Nanoscale BioPhotonics (CNBP), The University of Adelaide, South Australia, Australia 5005

<sup>c</sup> School of Chemical Sciences, The University of Auckland, 23 Symonds Street, Auckland 1010, New Zealand.

<sup>d</sup> Maurice Wilkins Centre for Molecular Biodiscovery, The University of Auckland, Private Bag 92019, Auckland 1010, New Zealand.

<sup>e</sup> Institute for Innovation in Biotechnology, The University of Auckland, 3A Symonds Street, Auckland 1010, New Zealand.

<sup>f</sup> School of Molecular and Biomedical Sciences, The University of Adelaide, Adelaide 5005, SA, Australia.

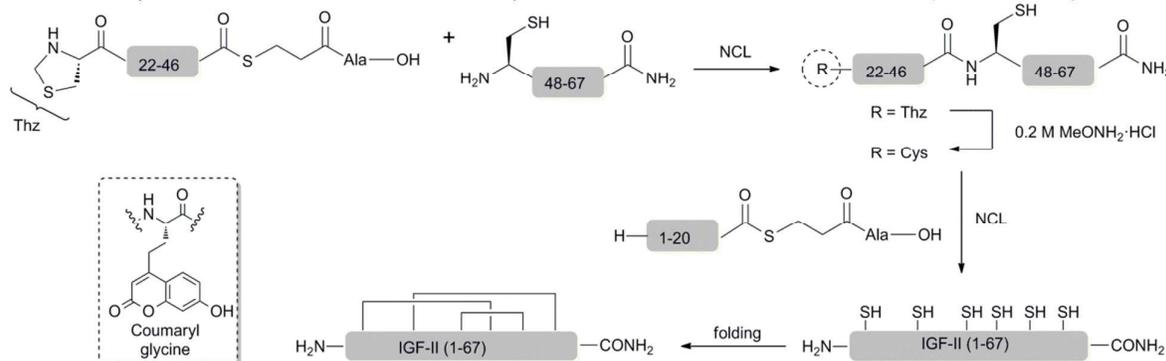
<sup>g</sup> School of Medicine, Flinders University of South Australia, Bedford Park, 5042, SA, Australia.

† Footnotes relating to the title and/or authors should appear here.

Electronic Supplementary Information (ESI) available: Synthesis and characterisation of peptide fragments, experimental conditions, analysis of ligations and fluorescent experiments are described. See DOI: 10.1039/x0xx00000x

(based on the starting resin). Thus a more modular approach to the coumaryl glycine-containing IGF-II analogues was deemed necessary. With this in mind we have developed an

efficient convergent synthesis of IGF-II and its application to the preparation of two specific fluorescent IGF-II analogues; F19Cou IGF-II and F28Cou IGF-II (see Scheme 1).



Scheme 1: One-pot, three fragment ligation approach to the synthesis of IGF-II analogues. Inset: coumaryl glycine acceptor. NCL conditions: 6.0 M GnHCl, 200.0 mM Na<sub>2</sub>HPO<sub>4</sub>, 20.0 mM TCEP, 200.0 mM MPAA, pH of 6.7–7.0. Folding conditions: 2.50 M urea, 0.70 M Tris, 12.5 mM glycine, 2.0 mM EDTA, 0.5 mM DTT, 1.25 mM 2-hydroxyethyl disulphide at a pH of 9.1 and protein concentration of <0.10 mg/mL.

## Results and discussion

The six Cys residues (Cys<sup>9</sup>, Cys<sup>21</sup>, Cys<sup>46</sup>, Cys<sup>47</sup>, Cys<sup>51</sup> and Cys<sup>60</sup>) of the IGF's provide suitable sites for a native chemical ligation (NCL) approach to the synthesis.<sup>48</sup> The viability of such an approach to IGF-II and its analogues was first investigated with a two fragment-based synthesis of native IGF-II. This began with the synthesis of a C-terminal IGF-II (47-67) fragment by standard Fmoc-SPPS and the N-terminal IGF-II (1-46) thioester using an *in situ* neutralisation Boc-SPPS protocol (see Supporting Information).<sup>49</sup> Ligation of the N-terminal IGF-II (1-46) thioester and the C-terminal IGF-II (47-67) fragment, in the presence of 6.0 M GnHCl, 200 mM Na<sub>2</sub>HPO<sub>4</sub>, 20 mM TCEP, 200 mM MPAA<sup>50</sup>, at a pH of 6.8, gave the desired native IGF-II peptide (see Supporting Information). However, access to sufficient quantities of the required 46-residue fragment, or unnatural amino acid-containing derivatives thereof, proved impractical due to low yields associated with the assembly and purification of such a long peptide sequence. Therefore, a more convergent, three fragment-based approach to synthesis of the native IGF-II and fluorescent IGF-II analogues (F19Cou IGF-II and F28Cou IGF-II) was investigated as summarised in Scheme 1.

The IGF-II peptides were assembled from three fragments of 20, 26 and 21 amino acids in length respectively, using iterative Val<sup>20</sup> to Cys<sup>21</sup>, and Cys<sup>46</sup> to Cys<sup>47</sup> ligations as depicted in Scheme 1. Specific ligation sites were selected based on the size of the respective fragments and the predicted reactivity of the C-terminal residue bearing the thioester.<sup>51</sup> Thus the Val<sup>20</sup>-Cys<sup>21</sup> junction (rather than Leu<sup>8</sup>-Cys<sup>9</sup>) was selected as the N-terminal ligation site, where disconnection here gives rise to peptide fragments of similar size (approx. 20 residues) (see Supporting Information for IGF-II sequence).

The peptide thioesters (IGF-II (1-20) and IGF-II (Thz-46)) were prepared using an *in situ* neutralisation Boc-SPPS protocol,<sup>49</sup> and the C-terminal IGF-II (47-67) fragment was synthesised using standard Fmoc-SPPS (see Supporting Information). The

use of a manual *in situ* neutralisation Boc-SPPS protocol for the preparation of the N-terminal IGF-II (1-20) thioester prevented the formation of deletion by-products, which resulted from on-resin aggregation using an Fmoc-SPPS approach.<sup>47</sup> The use of TFA in the deprotection of the N $\alpha$ -Boc amino functionality was key in disrupting on-resin aggregation.<sup>52, 53</sup>

The one-pot three fragment synthesis of the native IGF-II peptide (summarised in Figure 1) began with ligation of the IGF-II (Thz-46) thioester with the C-terminal IGF-II (47-67) fragment under standard NCL conditions.<sup>50, 54</sup> LCMS analysis of the reaction mixture, after 60 min, revealed complete conversion to the IGF-II (Thz-67) fragment (see Figure 1b). Treatment of this ligation mixture with methoxyamine hydrochloride (0.2 M) at pH 3,<sup>55</sup> for 8 h gave complete conversion (as determined by LCMS) of the thiazolidone (Thz) to cysteine (refer to Figure 1c). The ligation mixture was then returned to pH 6.8 and the N-terminal IGF-II (1-20) thioester was added. Complete conversion to the desired native IGF-II peptide was apparent after 36 h, based on LCMS analysis as shown in Figure 1d, despite the expected slow reactivity of Val thioester.<sup>51</sup> Native IGF-II peptide was isolated by solid phase extraction and purified by RP-HPLC. This material was then folded using the optimised folding conditions described by Delaine *et al.*<sup>56</sup> (see Supporting Information) to give synthetic native IGF-II in an overall yield of 0.3% (from the IGF-II (Thz-67) thioester fragment) and importantly in improved purity (>98% based on RP-HPLC, see Figure 2b) compared to previous reports.<sup>44, 45</sup>

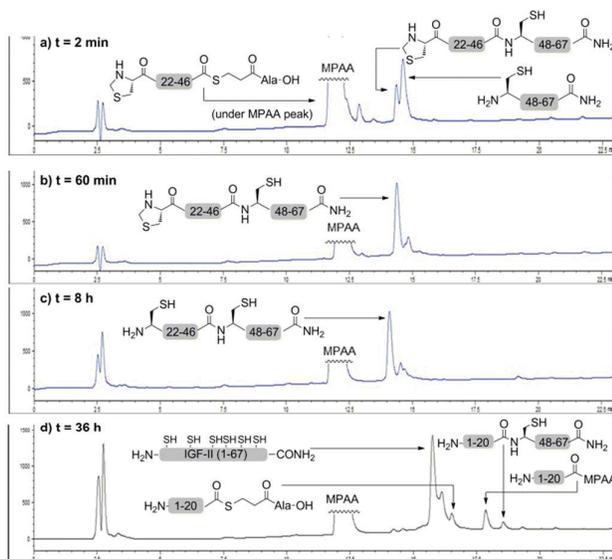


Figure 1: LCMS analysis of the one-pot three fragment synthesis of the native IGF-II peptide. Analysis of the cysteine-based ligation between the IGF-II (Thz-46) thioester and C-terminal IGF-II (47-67) fragment after a) 2 min and b) 60 min; c) Analysis of the thiazolidone deprotection of the IGF-II (Thz-67) fragment after 8 h; d) Analysis of the valine-based ligation between the N-terminal IGF-II (1-20) thioester and IGF-II (21-67) fragment after 36 h.

The methodology was next applied to the synthesis of the two fluorescent IGF-II analogues, F19Cou IGF-II and F28Cou IGF-II. The F19Cou IGF-II peptide was synthesised by ligation of the N-terminal F19Cou IGF-II (1-20) thioester, IGF-II (Thz-46) thioester and C-terminal IGF-II (47-67) fragment. The F28Cou IGF-II peptide was similarly synthesised by ligation of the N-terminal IGF-II (1-20) thioester, F28Cou IGF-II (Thz-46) thioester and C-terminal IGF-II (47-67) fragment. Folding of the fluorescent IGF-II peptides was essentially as described above for the native IGF-II protein, and resulted in a single folded product (see Supporting Information).<sup>56</sup> RP-HPLC was used to confirm the correct folding of the synthetic IGF-II analogues, which was consistent with folding profiles observed for other IGF-II analogues.<sup>33, 57</sup> Both the F19Cou IGF-II and F28Cou IGF-II proteins were isolated in excellent purity (>98% based on RP-HPLC, see Figure 2c-d), and in moderate to low overall yields of 1% (from the IGF-II (Thz-67) thioester fragment) and 0.1% (from the F28Cou IGF-II (Thz-67) thioester fragment) respectively. A real advantage of the ligation methodology is that it is robust and highly reproducible. Sufficient quantities of peptide were obtained for the study, however the yield from protein folding could likely be improved if conducted on a larger scale.

Competition binding assays of the synthetic IGF-II analogues with the IGF-1R were conducted using solubilized immunocaptured IGF-1R<sup>58</sup> and the resulting binding curves are depicted in Figure 3. The  $IC_{50}$  values for the binding of the synthetic native IGF-II (two fragment synthesis) and synthetic native IGF-II (three fragment synthesis), to the IGF-1R were determined to be  $2.1 \pm 1.6$  nM and  $2.0 \pm 1.2$  nM respectively, see Table 1. These values are essentially identical to the  $IC_{50}$  values determined and those reported for the recombinant

native IGF-II protein ( $2.1 \pm 1.2$  nM).<sup>56</sup> The F19Cou IGF-II and the F28Cou IGF-II proteins gave  $IC_{50}$  values of  $7.0 \pm 1.3$  nM and  $6.5 \pm 1.5$  nM respectively. A small decrease in affinity compared to the native IGF-II protein was not unexpected since both Phe<sup>19</sup> and Phe<sup>28</sup> are reported to be important residues for IGF-II binding.<sup>33</sup> Importantly, the observed three-fold decrease in binding affinity is consistent with reports for other IGF-II analogues with substitutions at Phe<sup>19</sup> and Phe<sup>28</sup>.<sup>33, 56</sup>

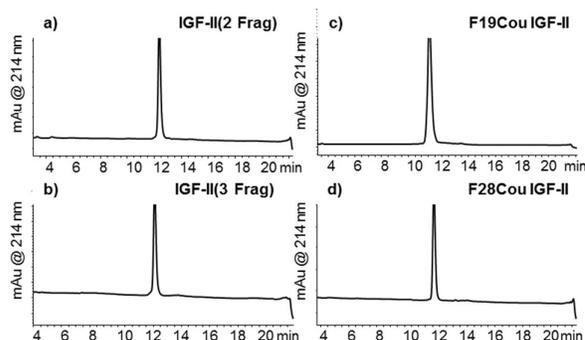


Figure 2: Purified synthetic IGF-II proteins synthesised using NCL approach. a) native IGF-II (2 Fragment); b) native IGF-II (3 Fragment); c) F19Cou IGF-II; d) F28Cou IGF-II.

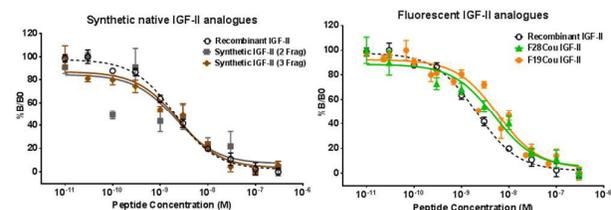


Figure 3: Competitive binding of the synthetic native IGF-II, F19Cou IGF-II, and F28Cou IGF-II proteins to immunocaptured IGF-1R. Immunocaptured IGF-1R was incubated with europium-labelled IGF-II (EuIGF-II) in the absence or presence of increasing concentrations of recombinant native IGF-II (black, dashed line), synthetic native IGF-II (two fragment: grey squares; three fragment: brown diamonds), F19Cou IGF-II (orange circles) or F28Cou IGF-II (green triangles). Results are expressed as a percentage of binding in the absence of competing ligand (Bo). Graphs show data pooled from three separate experiments and each data point is measured in triplicate per experiment, except data from the native IGF-II protein (two fragment) was derived from a single experiment performed in triplicate. Data is shown as the mean  $\pm$  S.E. Error bars are shown when greater than the size of the symbols.

Table 1:  $IC_{50}$  values derived from competitive binding assays of the synthetic IGF-II analogues binding to immunocaptured IGF-1R. Where the affinity relative to IGF-II is the  $IC_{50}$  relative to that of IGF-II binding to the IGF-1R ( $IC_{50}$  IGF-II /  $IC_{50}$  IGF-II analogue) and is expressed as a percentage of IGF-II binding. IGF-II  $\pm$  S.E. is derived from at least three separate experiments performed in triplicate

Protein	$IC_{50}$ (nM)	Affinity relative to recombinant native IGF-II (%)
recombinant native IGF-II	$2.1 \pm 1.2$	100
native IGF-II (2 fragment) <sup>[a]</sup>	$2.1 \pm 1.6$	100
native IGF-II (3 fragment)	$2.0 \pm 1.2$	105
F19Cou IGF-II	$7.0 \pm 1.3$	30
F28Cou IGF-II	$6.5 \pm 1.5$	32

[a] results derived from a single experiment performed in triplicate

A FRET analysis of the binding of both fluorescent IGF-II analogues to a soluble form of the IGF-1R (sIGF-1R)<sup>59</sup> was next investigated. Native tryptophan fluorescence from the IGF-1R was used as the donor, with the coumarin contained within synthetic F19Cou IGF-II and F28Cou IGF-II proteins acting as an acceptor. Binding was investigated using a sensitized emission approach, where the presence of a FRET signal requires the tryptophan fluorescence of the IGF-1R to be quenched and the coumarin fluorescence of the IGF-II analogue is sensitized. The experiment involved titration of an IGF-II analogue (acceptor) into a solution of sIGF-1R (donor) until the protein and receptor were present in an equimolar ratio. After each addition of an IGF-II analogue, the sample was excited at 280 nm and 320 nm and the fluorescence emission spectrum for each wavelength was recorded (see Supplementary Information). Control experiments for the IGF-II analogues (acceptor) and sIGF-1R (donor) were also analysed using the same methodology.

The resulting fluorescence emission spectra for the native IGF-II, F19Cou IGF-II, and F28Cou IGF-II, after excitation at 280 nm and 320 nm are shown in **Error! Reference source not found.** Figure 4, Figure 5 **Error! Reference source not found.** and Figure 6 **Error! Reference source not found.** respectively. Specifically fluorescence emission spectra of the IGF-II analogues (acceptor) in the absence and presence of the donor (sIGF-1R), the emission of the donor (sIGF-1R) and the summed emission spectrum resulting from the sum of the individual donor and acceptor spectra are displayed. The spectra reveal two key emissions, one at 332 nm resulting from excitation of the sIGF-1R (donor) and a second at 455 nm that arises from excitation of the coumarin within the synthetic F19Cou IGF-II and F28Cou IGF-II analogues (acceptor).

The fluorescence emission spectra for the native IGF-II analogue are shown in Figure 4 **Error! Reference source not found.** As expected, excitation at 280 nm of native IGF-II (control) alone (Figure 4A: purple solid line) gave no fluorescence emission at 332 nm. This is because native IGF-II lacks endogenous Trp or a fluorescent probe. Similarly, excitation at 320 nm of native IGF-II alone (Figure 4 **Error! Reference source not found.** B: purple solid line) did not result in an increase in fluorescence emission at 455 nm as it lacks the coumarin fluorophore present in F19Cou IGF-II and F28Cou IGF-II. However, excitation at 280 nm of the sIGF-1R (donor) alone gave an intense emission at 332 nm (Figure 4 **Error! Reference source not found.** A: red solid line), which is due to the presence of endogenous Trp. Surprisingly, excitation at 280 nm of the sIGF-1R (donor) in the presence of native IGF-II resulted in fluorescence emission at 332 nm that was less intense than the fluorescence emission at 332 nm for the sIGF-1R alone (Figure 4 **Error! Reference source not found.** A: red solid line). This emission at 332 nm was also less intense than the sum of the individual emission spectra of native IGF-II and sIGF-1R (Figure 4 **Error! Reference source not found.** A: blue solid line). This large decrease in fluorescence at 332 nm indicates that the receptor (sIGF-1R) is extremely sensitive to ligand binding. The decrease is likely the result of extensive Trp

quenching within the sIGF-1R, or perhaps a decrease in the quantum yield of the endogenous Trp within the sIGF-1R. This is an interesting result that suggests a change in the local environment of the IGF-1R Trp residues upon IGF-II binding, and supports the idea of a structural change in the receptor upon ligand binding.

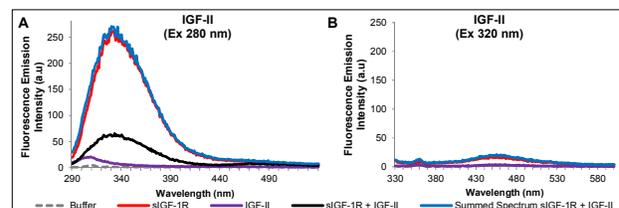


Figure 4: Summary of fluorescence emission spectra of the native IGF-II analogue and sIGF-1R after excitation at 280 nm (A) and 320 nm (B). Fluorescence emission spectra in the absence (purple solid line) and presence (black solid line) of the sIGF-1R, the emission of the sIGF-1R (red solid line) and the summed emission spectrum resulting from the sum of the individual donor (sIGF-1R) and acceptor (native IGF-II) spectra (blue solid lines) are displayed. Spectra were collected when the protein and receptor were present in an equimolar ratio at concentration of 0.20  $\mu\text{M}$ , in 0.1 M sodium phosphate buffer at pH 7.2 after excitation at 280 nm or 320 nm. Spectra are derived from a single experiment, where each spectrum is averaged from three consecutive scans and have not been corrected for background fluorescence.

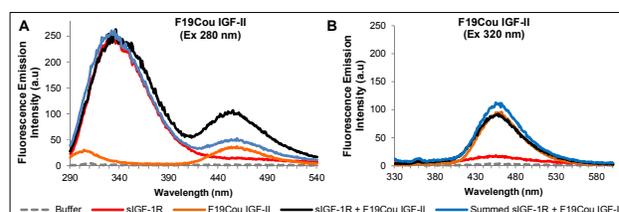


Figure 5: Summary of fluorescence emission spectra of the F19Cou IGF-II analogue after excitation at 280 nm (A) and 320 nm (B). Fluorescence emission spectra in the absence (orange solid line) and presence (black solid line) of the sIGF-1R, the emission of the sIGF-1R (red solid line) and the summed emission spectrum resulting from the sum of the individual donor (sIGF-1R) and acceptor (F19Cou IGF-II) spectra (blue solid lines) are displayed. Spectra were collected when the protein and receptor were present in an equimolar ratio at concentration of 0.20  $\mu\text{M}$ , in 0.1 M sodium phosphate buffer at pH 7.2 after excitation at 280 nm or 320 nm. Spectra are derived from a single experiment, where each spectrum is averaged from three consecutive scans and have not been corrected for background fluorescence.

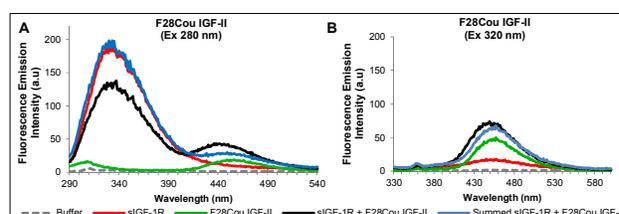


Figure 6: Summary of fluorescence emission spectra of the F28Cou IGF-II analogue after excitation at 280 nm (A) and 320 nm (B). Fluorescence emission spectra in the absence (green solid line) and presence (black solid line) of the sIGF-1R, the emission of the sIGF-1R (red solid line) and the summed emission spectrum resulting from the sum of the individual donor (sIGF-1R) and acceptor (F28Cou IGF-II) (blue solid lines) are displayed. Spectra were collected when the protein and receptor were present in an equimolar ratio at concentration of 0.19  $\mu\text{M}$ , in 0.1 M sodium phosphate buffer at pH 7.2 after excitation at 280 nm or 320 nm. Spectra are derived from a single experiment, where each spectrum is averaged from three consecutive scans and have not been corrected for background fluorescence.

An analysis of the fluorescence emission spectra for the F19Cou IGF-II analogue alone and in complex with the sIGF-1R is shown in Figure 5. Excitation at 280 nm of the F19Cou IGF-II analogue alone (Figure 5A: orange solid line) resulted in no fluorescence emission at 332 nm and a small intensity emission at 455 nm, and excitation of the sIGF-1R alone (donor) at 280 nm produced an intense emission peak at 332 nm (Figure 5A: red solid line). Excitation at 280 nm of F19Cou IGF-II (acceptor) in the presence of the sIGF-1R (donor) (Figure 5A: black solid line) resulted in fluorescence emissions at 332 nm and 455 nm. The fluorescence emission of the complex at 455 nm was increased compared to the fluorescence emission for the acceptor (F19Cou IGF-II) alone (Figure 5A: orange solid line). Whereas the fluorescence emission of the complex at 332 nm was unchanged compared to the fluorescence emission spectrum for the donor (sIGF-1R) alone (Figure 5A: red solid line), and was unchanged compared to the sum of the individual emission spectra of the sIGF-1R and F19Cou IGF-II (Figure 5A: blue solid line). This unaltered fluorescence emission at 332 nm suggests a lack of Trp quenching in the IGF-1R upon F19Cou IGF-II binding. Excitation at 320 nm of the F19Cou IGF-II (acceptor) alone (Figure 5B: orange solid line) gave rise to fluorescence emission at 455 nm. The same fluorescence emission at 455 nm was also observed for the F19Cou IGF-II in complex with the sIGF-1R (acceptor & donor) (Figure 5B: black solid line), after excitation at 320 nm. Importantly the fluorescence emission at 455 nm for F19Cou IGF-II in complex with the sIGF-1R (acceptor & donor) was identical in intensity to the emission for the F19Cou IGF-II alone (acceptor). These results demonstrate that fluorescence emission at 455 nm is enhanced upon F19Cou IGF-II binding to the receptor (sIGF-1R), and direct excitation (Ex 320 nm) of the coumarin residue is unaffected after ligand binding. Together these results confirm the F19Cou IGF-II coumarin fluorescence is sensitized. Figure 7 shows the acceptor emission data from the FRET interaction between F19Cou IGF-II and the sIGF-1R. Specifically, the extracted acceptor emission (Figure 7: black dotted line) and the extracted FRET signal (Figure 7: blue dotted line) are shown. Where the extracted acceptor emission (Figure 7: black dotted line) was obtained by subtracting the donor (sIGF-1R) emission from the complex (sIGF-1R and F19Cou IGF-II) emission, and the FRET signal (Figure 7: blue dotted line) was obtained by subtracting the donor (sIGF-1R) and acceptor (F19Cou IGF-II) spectra from the complex (sIGF-1R and F19Cou IGF-II) spectra. The extracted acceptor emission (Figure 7: black dotted line) shows a fluorescence emission at 455 nm, which is consistent with coumarin emission. The emission was more intense than the fluorescence emission at 455 nm for the acceptor (F19Cou IGF-II) alone (Figure 7: orange solid line). This increase in fluorescence emission at 455 nm is also visible in the extracted FRET signal shown in Figure 7 (blue dotted line). Together these results suggest that the quantum yield of the Trp residues, within the IGF-1R, is enhanced by F19Cou IGF-II binding but is simultaneously and equally quenched by FRET.

This results in the unaltered Trp emission seen in Figure 5. Error! Reference source not found..

Figure 6 depicts the FRET analysis of the F28Cou IGF-II alone and in complex with the sIGF-1R. Excitation at 280 nm of F28Cou IGF-II alone (acceptor) (Figure 6A: solid green line) gave a low intensity fluorescence emission at 455 nm, and excitation at 280 nm of the F28Cou IGF-II in complex with sIGF-1R (donor & acceptor) (Figure 6A: black solid line), gave fluorescence emissions at 332 nm and 455 nm. The fluorescence emission at 332 nm was less intense than the emission at 332 nm for the sIGF-1R alone (Figure 6A: red solid line). While the fluorescence emission at 455 nm for the complex was more intense than the emission at the same wavelength for the acceptor (F28Cou IGF-II) alone (Figure 6A: green solid line).

The relative decrease in fluorescence at 332 nm between the complex and receptor alone is consistent with Trp quenching within the sIGF-1R upon F28Cou IGF-II binding, albeit to a lesser extent than with the native IGF-II (Figure 4). While the increase in fluorescence emission at 455 nm for the complex compared to the acceptor (F28Cou IGF-II) alone, is consistent with a FRET interaction between the sIGF-1R and F28Cou IGF-II. Furthermore excitation at 320 nm gave fluorescence emission at 455 nm for both the F28Cou IGF-II in complex with the sIGF-1R (acceptor & donor) (Figure 6B: black solid line) and F28Cou IGF-II alone (acceptor) (Figure 6B: green solid line). This emission was only slightly more intense for the F28Cou IGF-II in complex with the sIGF-1R than for the F28Cou IGF-II alone, and suggests direct excitation (Ex 320 nm) of the coumarin residue is relatively unaffected after ligand binding. These results are supported by the acceptor emission data shown in Figure 8.

The extracted acceptor emission shown in Figure 8 gave fluorescence emission at 455 nm, which is consistent with acceptor emission. This extracted emission was more intense than the fluorescence emission at 455 nm for the acceptor (F28Cou IGF-II) alone (Figure 8: orange solid line). This emission is also shown in the extracted FRET signal (Figure 8: blue dotted line). Together this data confirms the increase in the fluorescence emission at 455 nm is likely the result of a FRET interaction between the Trp residues of the IGF-1R and the coumarin of the F28Cou IGF-II analogue. A comparison of F19Cou IGF-II and F28Cou IGF-II acceptor emission spectra shown in Figure 7 and Figure 8 (blue dotted lines) shows the FRET signal for F19Cou IGF-II is stronger than the FRET signal for the F28Cou IGF-II analogue. This suggests that binding of F28Cou IGF-II to the sIGF-1R causes a decrease in the quantum yield of the receptor (sIGF-1R), leading to a decrease in fluorescence emission at 332 nm, which results in the reduced emission at 332 nm shown in Figure 6.

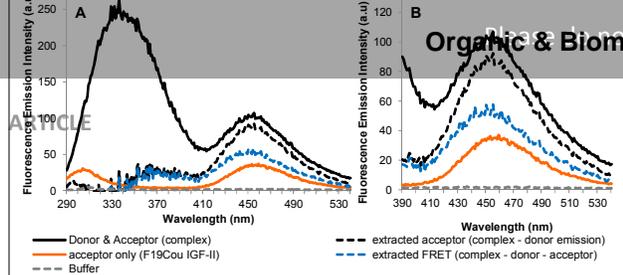


Figure 7: Sensitized fluorescence emission spectra for the F19Cou IGF-II in complex with the sIGF-1R after excitation at 280 nm (A) with expansion between 390–540 nm (B). Fluorescence emission spectra of the complex (sIGF-1R and F19Cou IGF-II) uncorrected (black solid line), extracted acceptor emission (black dotted line) which was obtained by subtracting the donor (sIGF-1R) emission from the complex (sIGF-1R and F19Cou IGF-II) emission, uncorrected acceptor (F19Cou IGF-II) emission (orange solid line), corrected acceptor (F19Cou IGF-II) emission (orange dotted line), which was obtained by subtracting the donor (sIGF-1R) emission from the acceptor (F19Cou IGF-II) emission spectrum, fluorescence emission spectrum resulting from FRET (blue dotted line), which was obtained by subtracting the donor (sIGF-1R) and acceptor (F19Cou IGF-II) spectra from the complex (sIGF-1R and F19Cou IGF-II) are displayed. Spectra were collected when the protein and receptor were present in an equimolar ratio at concentration of 0.19  $\mu\text{M}$ , in 0.1 M sodium phosphate buffer at pH 7.2 after excitation at 280 nm. Spectra are derived from a single experiment, where each spectrum is averaged from three consecutive scans.

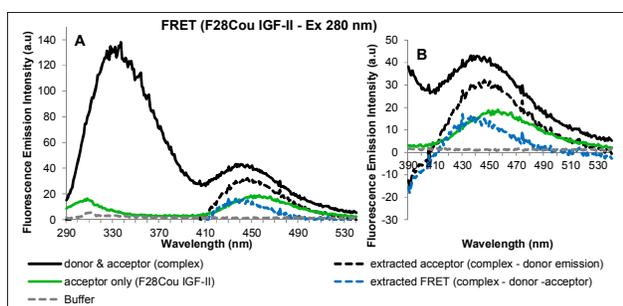


Figure 8: Sensitized fluorescence emission spectra for the F28Cou IGF-II in complex with the sIGF-1R after excitation at 280 nm (A) with expansion between 390–540 nm (B). Fluorescence emission spectra of the complex (sIGF-1R and F28Cou IGF-II) uncorrected (black solid line), extracted acceptor emission (black dotted line) which was obtained by subtracting the donor (sIGF-1R) emission from the complex (sIGF-1R and F28Cou IGF-II) emission, uncorrected acceptor (F28Cou IGF-II) emission (green solid line), corrected acceptor (F28Cou IGF-II) emission (green dotted line), which was obtained by subtracting the donor (sIGF-1R) emission from the acceptor (F28Cou IGF-II) emission spectrum, fluorescence emission spectrum resulting from FRET (blue dotted line), which was obtained by subtracting the donor (sIGF-1R) and acceptor (F28Cou IGF-II) spectra from the complex (sIGF-1R and F28Cou IGF-II) are displayed. Spectra were collected when the protein and receptor were present in an equimolar ratio at concentration of 0.19  $\mu\text{M}$ , in 0.1 M sodium phosphate buffer at pH 7.2 after excitation at 280 nm. Spectra are derived from a single experiment, where each spectrum is averaged from three consecutive scans.

A FRET interaction was expected for both site 1 (F28Cou IGF-II) and site 2 (F19Cou IGF-II) interactions as there are several naturally occurring Trp residues located adjacent to the putative IGF-II binding sites (including Trp residues 79, 127, 176, 244 for site 1 and 402, 404, 479, 519, 601 and 618 for site 2) (see Supporting Information).<sup>19, 27, 28, 43</sup> Of these, the most likely donor candidates, identified from the X-ray crystallographic data of the insulin:IR interaction<sup>19, 31</sup> are Trp79, Trp519 and Trp544, as these residues are surface exposed and located approximately 18–25 Å from the proposed ligand binding site. As expected, an increase in fluorescence emission was not observed for the native IGF-II protein in the presence of the sIGF-1R, as it lacks a fluorescent probe (**Error! Reference source not found.**). However surprisingly binding of native IGF-II to the sIGF-1R causes a

significant decrease in the Trp fluorescence of the sIGF-1R. The site 2, F19Cou IGF-II analogue (**Error! Reference source not found.**) displayed fluorescence emission that was sensitized, and Trp emission was unaltered. Finally, the F28Cou IGF-II analogue displayed Trp fluorescence which was lower in the presence of the sIGF-1R and the coumarin fluorescence of the F28Cou IGF-II analogue was sensitized. These results confirm that the coumarin probes of the F19Cou IGF-II and F28Cou IGF-II bind in close proximity (10–100 Å) to a Trp residue(s) within the IGF-1R and in turn Phe19 and Phe28 are appropriate sites within IGF-II for the incorporation of a fluorescent probe/FRET acceptor. The strong binding affinities and positive FRET results demonstrate these are good analogues for further FRET binding studies.

## Conclusions

In conclusion, we report an efficient, modular synthesis of the native IGF-II protein and two fluorescent IGF-II analogues. A three fragment approach using a Val20-Cys21 ligation site, gave complete ligation in less than 47 h. The IGF-II proteins were isolated in comparable yields (0.1–1%) and importantly in higher purity compared to previously reported IGF-II syntheses (c.a. 2%).<sup>44–46</sup> The native IGF-II and two fluorescent IGF-II analogues bind with nanomolar affinity to the IGF-1R. Both the F19Cou IGF-II and F28Cou IGF-II proteins displayed a FRET interaction with the IGF-1R, with binding of the native IGF-II protein causing the largest quenching of IGF-1R fluorescence. These results demonstrate that the Trp residues within the sIGF-1R are extremely sensitive to ligand binding, and these coumarin probes bind in close proximity to Trp residues within the IGF-1R and as such are ideal analogues for investigating the interaction of IGF-II with its high affinity binding partners.

## Acknowledgements

The authors would like to acknowledge Ms Carlie Delaine for preparation of the sIGF-1R and Ms Clair Alvino for helpful discussions. The research was supported in part by the ARC Centre of Excellence in Nanoscale BioPhotonics (CNBP).

## Notes and references

1. S. Kornfeld, *Annual Review of Biochemistry*, 1992, **61**, 307–330.
2. E. Van Obberghen, *Diabetologia*, 1994, **37**, S125–S134.
3. C. E. H. Stewart and P. Rotwein, *Physiol. Rev.*, 1996, **76**, 1005–1026.
4. A. Denley, L. J. Cosgrove, G. W. Booker, J. C. Wallace and B. E. Forbes, *Cytokine Growth Factor Rev.*, 2005, **16**, 421–439.
5. A. Belfiore, F. Frasca, G. Pandini, L. Sciacca and R. Vigneri, *Endocrine Reviews*, 2009, **30**, 586–623.

6. L. K. Harris and M. Westwood, *Growth Factors*, 2012, **30**, 1-12.
7. H. Yu and T. Rohan, *Journal of the National Cancer Institute*, 2000, **92**, 1472-1489.
8. M. N. Pollak, E. S. Schernhammer and S. E. Hankinson, *Nature Reviews Cancer*, 2004, **4**, 505-518.
9. A. A. Samani, S. Yakar, D. LeRoith and P. Brodt, *Endocrine Reviews*, 2007, **28**, 20-47.
10. F. Frasca, G. Pandini, L. Sciacca, V. Pezzino, S. Squatrito, A. Belfiore and R. Vigneri, *Archives Of Physiology And Biochemistry*, 2008, **114**, 23-37.
11. M. Pollak, *Nature Reviews Cancer*, 2008, **8**, 915-928.
12. E. J. Gallagher and D. LeRoith, *Endocrinology*, 2011, **152**, 2546-2551.
13. A. Belfiore and R. Malaguarnera, *Endocrine-Related Cancer*, 2011, **18**, R125-R147.
14. J. Gao, Y. S. Chang, B. Jallal and J. Viner, *Cancer Research*, 2012, **72**, 3-12.
15. M. Pollak, *Clinical Cancer Research*, 2012, **18**, 40-50.
16. J. Brown, E. Y. Jones and B. E. Forbes, in *Vitamins & Hormones*, ed. L. Gerald, Academic Press, 2009, vol. Volume 80, pp. 699-719.
17. H. M. El-Shewy and L. M. Luttrell, in *Vitamins & Hormones*, ed. L. Gerald, Academic Press, 2009, vol. Volume 80, pp. 667-697.
18. J. G. Menting, Y. Yang, S. J. Chan, N. B. Phillips, B. J. Smith, J. Whittaker, N. P. Wickramasinghe, L. J. Whittaker, V. Pandeyarajan, Z.-I. Wan, S. P. Yadav, J. M. Carroll, N. Strokes, C. T. Roberts, F. Ismail-Beigi, W. Milewski, D. F. Steiner, V. S. Chauhan, C. W. Ward, M. A. Weiss and M. C. Lawrence, *Proceedings of the National Academy of Sciences*, 2014, **111**, E3395-E3404.
19. J. G. Menting, J. Whittaker, M. B. Margetts, L. J. Whittaker, G. K. W. Kong, B. J. Smith, C. J. Watson, L. Zakova, E. Kletvikova, J. Jiracek, S. J. Chan, D. F. Steiner, G. G. Dodson, A. M. Brzozowski, M. A. Weiss, C. W. Ward and M. C. Lawrence, *Nature*, 2013, **493**, 241-245.
20. T. P. J. Garrett, N. M. McKern, M. Lou, M. J. Frenkel, J. D. Bentley, G. O. Lovrecz, T. C. Elleman, L. J. Cosgrove and C. W. Ward, *Nature*, 1998, **394**, 395-399.
21. T. E. Adams and V. C. E. T. P. J. G. C. W. Ward, *Cellular and Molecular Life Sciences*, 2000, **57**, 1050-1093.
22. C. W. Ward, T. P. J. Garrett, N. M. McKern, M. Lou, L. J. Cosgrove, L. G. Sparrow, M. J. Frenkel, P. A. Hoyne, T. C. Elleman, T. E. Adams, G. O. Lovrecz, L. J. Lawrence and P. A. Tulloch, *Molecular Pathology*, 2001, **54**, 125-132.
23. P. De Meyts, *Bioessays*, 2004, **26**, 1351-1362.
24. V. C. Epa and C. W. Ward, *Protein Engineering Design and Selection*, 2006, **19**, 377-384.
25. M. Lou, T. P. J. Garrett, N. M. McKern, P. A. Hoyne, V. C. Epa, J. D. Bentley, G. O. Lovrecz, L. J. Cosgrove, M. J. Frenkel and C. W. Ward, *Proceedings of the National Academy of Sciences*, 2006, **103**, 12429-12434.
26. N. M. McKern, M. C. Lawrence, V. A. Streltsov, M.-Z. Lou, T. E. Adams, G. O. Lovrecz, T. C. Elleman, K. M. Richards, J. D. Bentley, P. A. Pilling, P. A. Hoyne, K. A. Cartledge, T. M. Pham, J. L. Lewis, S. E. Sankovich, V. Stoichevska, E. Da Silva, C. P. Robinson, M. J. Frenkel, L. G. Sparrow, R. T. Fernley, V. C. Epa and C. W. Ward, *Nature*, 2006, **443**, 218-221.
27. M. Keyhanfar, G. W. Booker, J. Whittaker, J. C. Wallace and B. E. Forbes, *Biochem. J.*, 2007, **401**, 269-277.
28. M. C. Lawrence, N. M. McKern and C. W. Ward, *Current Opinion in Structural Biology*, 2007, **17**, 699-705.
29. C. Ward, M. Lawrence, V. Streltsov, T. Garrett, N. McKern, M. Z. Lou, G. Lovrecz and T. Adams, *Acta Physiol.*, 2008, **192**, 3-9.
30. C. W. Ward and M. C. Lawrence, *Bioessays*, 2009, **31**, 422-434.
31. B. J. Smith, K. Huang, G. Kong, S. J. Chan, S. Nakagawa, J. G. Menting, S.-Q. Hu, J. Whittaker, D. F. Steiner, P. G. Katsoyannis, C. W. Ward, M. A. Weiss and M. C. Lawrence, *Proceedings of the National Academy of Sciences*, 2010, **107**, 6771-6776.
32. J. Whittaker, L. J. Whittaker, C. T. Roberts, N. B. Phillips, F. Ismail-Beigi, M. C. Lawrence and M. A. Weiss, *Proceedings of the National Academy of Sciences*, 2012.
33. C. L. Alvino, K. A. McNeil, S. C. Ong, C. Delaine, G. W. Booker, J. C. Wallace, J. Whittaker and B. E. Forbes, *J. Biol. Chem.*, 2009, **284**, 7656-7664.
34. R. M. Christie and C.-H. Lui, *Dyes and Pigments*, 1999, **42**, 85-93.
35. R. M. Christie and C.-H. Lui, *Dyes and Pigments*, 2000, **47**, 79-89.
36. B. Valeur and M. N. Berberan-Santos, *Molecular Fluorescence: Principles and Applications*, John Wiley & Sons, 2001.
37. J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, Springer, 3rd edn., 2006.
38. R. P. Haugland, *The Molecular Probes Handbook: A Guide to Fluorescent Probes and Labeling Technologies, 11th Edition*, Life Technologies, 2010.
39. L. M. Wysocki and L. D. Lavis, *Current Opinion in Chemical Biology*, 2011, **15**, 752-759.
40. J. Wang, J. Xie and P. G. Schultz, *Journal of the American Chemical Society*, 2006, **128**, 8738-8739.
41. M.-P. Brun, L. Bischoff and C. Garbay, *Angewandte Chemie International Edition*, 2004, **43**, 3432-3436.
42. L. Whittaker, C. Hao, W. Fu and J. Whittaker, *Biochemistry*, 2008, **47**, 12900-12909.
43. J. Whittaker, A. V. Groth, D. C. Mynarcik, L. Pluzek, V. L. Gadsboll and L. J. Whittaker, *J. Biol. Chem.*, 2001, **276**, 43980-43986.
44. C. H. Li, D. Yamashiro, R. Glenn Hammonds Jr and M. Westphal, *Biochem. Biophys. Res. Commun.*, 1985, **127**, 420-424.
45. D. Yamashiro and C. H. Li, *International Journal of Peptide and Protein Research*, 1985, **26**, 299-304.
46. Y. M. Oh, H. L. Muller, H. P. Zhang, N. Ling and R. G. Rosenfeld, in *Current Directions in Insulin-Like Growth Factor Research*, eds. D. LeRoith and M. K. Raizada,

- Plenum Press Div Plenum Publishing Corp, New York, 1993, vol. 343, pp. 41-54.
47. J. Cottam, D. Scanlon, J. Karas, A. Calabrese, T. Pukala, B. Forbes, J. Wallace and A. Abell, *International Journal of Peptide Research and Therapeutics*, 2013, **19**, 61-69.
48. Y. Sohma, B. L. Pentelute, J. Whittaker, Q.-x. Hua, L. J. Whittaker, M. A. Weiss and S. B. H. Kent, *Angewandte Chemie International Edition*, 2008, **47**, 1102-1106.
49. M. Schnölzer, P. Alewood, A. Jones, D. Alewood and S. B. H. Kent, *International Journal of Peptide and Protein Research*, 1992, **40**, 180-193.
50. E. C. B. Johnson and S. B. H. Kent, *Journal of the American Chemical Society*, 2006, **128**, 6640-6646.
51. T. M. Hackeng, J. H. Griffin and P. E. Dawson, *Proceedings of the National Academy of Sciences*, 1999, **96**, 10068-10073.
52. R. C. d. L. Milton, S. C. F. Milton and P. A. Adams, *Journal of the American Chemical Society*, 1990, **112**, 6039-6046.
53. M. Schnölzer, P. Alewood, A. Jones, D. Alewood and S. Kent, *International Journal of Peptide Research and Therapeutics*, 2007, **13**, 31-44.
54. P. Dawson, T. Muir, I. Clark-Lewis and S. Kent, *Science*, 1994, **266**, 776-779.
55. M. Villain, J. Vizzavona and K. Rose, *Chemistry & biology*, **8**, 673-679.
56. C. Delaine, C. L. Alvino, K. A. McNeil, T. D. Mulhern, L. Gauguin, P. De Meyts, E. Y. Jones, J. Brown, J. C. Wallace and B. E. Forbes, *J. Biol. Chem.*, 2007, **282**, 18886-18894.
57. C. L. Alvino, S. C. Ong, K. A. McNeil, C. Delaine, G. W. Booker, J. C. Wallace and B. E. Forbes, *PLoS One*, 2011, **6**.
58. A. Denley, E. R. Bonython, G. W. Booker, L. J. Cosgrove, B. E. Forbes, C. W. Ward and J. C. Wallace, *Mol. Endocrinol.*, 2004, **18**, 2502-2512.
59. K. H. Surinya, B. E. Forbes, F. Occhiodoro, G. W. Booker, G. L. Francis, K. Siddle, J. C. Wallace and L. J. Cosgrove, *J. Biol. Chem.*, 2008, **283**, 5355-5363.